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Investigations into the role of substance P and the NK-1 receptor in an animal model of neuropathic pain

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ABSTRACT

This study investigates the role of substance P and NK-1 receptors in an animal model of neuropathic pain. Unlike naïve animals, innocuous peripheral stimulation of neuropathic animals was determined to cause heterosegmental inhibition in the tail-flick test as well as increased plasma extravasation in the paw. These effects were prevented by administration of CP-96,345 before stimulation. Additionally, CP-96,345 or an antisense oligonucleotide against NK-1 receptors significantly alleviated mechanical allodynia in neuropathic animals. Finally, mass spectrum of lumbar spinal cord of neuropathic but not naïve animals showed a significant upregulation of substance P. We conclude that innocuous stimulation of a neuropathic area could trigger activation of NK-1 receptors, presumably due to binding of substance P. Furthermore, this activation of NK-1 receptors could be central to the perception of mechanical allodynia in neuropathic pain. These results justify the investigation of inhibiting the interaction between substance P and the NK-1 receptor for the treatment of drug resistant neuropathies.

RÉSUMÉ

Ce projet étudie le rôle de la substance P et des récepteurs NK-1 dans un model animal de douleur neuropathique. Contrairement aux animaux naïfs, une stimulation périphérique non-douloureuse des animaux neuropathiques entraîne une inhibition hétérosegmentale dans le test de rétraction de la queue ainsi qu'une augmentation de l'extravasation plasmatique dans la patte. Ces effets furent bloqués par l'administration de CP-96,345 avant la stimulation. De plus, CP-96,345 ainsi qu'un oligonucléotide anti-sens contre le récepteur NK-1 a atténué de façon significative l'allodynie mécanique chez les animaux neuropathiques. Finalement, nous avons démontré par spectroscopie de masse que contrairement aux animaux naïfs, la partie lombaire de la moelle épinière des animaux neuropathiques démontre une augmentation significative de la substance P. Ces résultats indiquent qu'une stimulation non-douloureuse d'une zone neuropathique peut provoquer l'activation des récepteurs NK-1, vraisemblablement du à la liaison de la substance P. De plus, l'activation des récepteurs de NK-1 pourrait jouer un rôle important dans la perception de l'allodynie mécanique lors de douleurs neuropathiques. Donc, cette étude justifie l'inhibition des interactions entre la substance P et le récepteur NK-1 pour le traitement de neuropathies résistantes aux médicaments.

CONTRIBUTION OF AUTHORS

The manuscript presented in this thesis represents the work of a number of individuals who are recognized for their contribution.

Brooks Fallis did the work demonstrating activation of NK-1 receptors following an innocuous stimulation of neuropathic animals both in the tail-flick test and in peripheral plasma extravasation. Brooks Fallis also examined the effect of an NK-1 receptor antagonist, morphine or gabapentin on mechanical allodynia in neuropathic animals. The data and manuscript included in this thesis was assembled and written by Brooks Fallis.

Dr. Catherine Cahill tested the effect of the NK-1 antisense oligonucleotide on alleviating mechanical allodynia in neuropathic animals. Dr. Cahill also performed the western blotting experiments and immunohistochemistry for confirmation of antisense efficacy.

Dr. Kiran Yashpal performed the tail-flick testing following intrathecal administration of substance P in antisense treated animals, and provided surgical assistance to Dr. Cahill.

Jennifer Ritchie tested the effect of intrathecally-administered verapamil, diltiazem methylene blue and phenytoin in neuropathic animals and performed neuropathic surgeries for the mass spectrum analysis.

Dr. Bernard Gibbs and Dr. Pascal Vachon, through MDS Pharma Services, did the mass spectrum analysis.

Dr. James Henry was the project supervisor and advisor to all listed above.

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TO DAD, MUM, JED, POD, ZO

AND

CHELSEA

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INTRODUCTION

Neuropathic pains occur in a variant group of chronic debilitating conditions caused by lesion or dysfunction of the nervous system. Patients describe a continuous burning pain, which in extreme cases makes walking or wearing clothes unbearable. Hyperalgesia (increased sensitivity to an already painful stimulus) and/or allodynia (pain sensation from a normally innocuous stimulus) and/or dysesthesia (spontaneous pain) can all occur in the affected area. One particular difficulty with neuropathic pains is that they occur in diseases of varied etiology and pathology. The prevalence of neuropathic pain can be conservatively estimated at 1.5% of the population of the United States (Bennett, 1998). The most commonly diagnosed neuropathic pains include neuropathic low back pains, diabetic neuropathy, postherpetic neuralgia, and reflex sympathetic dystrophy (Bennett, 1998).

Compounding the problem is the unusually limited success of traditional analgesics including powerful opioids such as morphine and NSAIDs such as ibuprofen in the alleviation of neuropathic pain (Arnér and Meyerson, 1988; Max et al., 1988; Kupers et al., 1991). Certain anti-convulsants such as gabapentin and some tricyclic anti-depressants including amitryptiline, were serendipitously discovered to provide a degree of analgesia to certain neuropathic pain patients. However, in addition to problems with tolerance, these drugs seem to either provide patients with miraculous relief, or are virtually useless (McQuay et al., 1996; Sindrup and Jensen, 1999). Other non-drug treatments used with varied efficacy to treat neuropathic pains include capsaicin cream, transcutaneous electrical nerve stimulation (TENS), physical and psychological therapies

and spinal cord stimulation. Despite the plethora of drugs and treatments available, many people with neuropathies remain in constant pain. As a result, neuropathic pain sufferers can experience severe depression. Clearly, neuropathic pain is an area of unmet medical need requiring new therapies.

A number of reliable animal models of neuropathic pain have been developed over the last fifteen years allowing the elucidation of some of the mechanisms behind the diseases. It seems there is no single occurrence that causes the painful state, but more likely a series of changes in the periphery, the spinal cord and higher brain centers initiated by a preliminary nerve injury. The following study further examines the role of substance P and the NK-1 receptor in neuropathic pain, in order to determine the importance of these peptides and elucidate novel therapeutic alternatives.

Substance P is an excitatory neuropeptide released from primary afferent neurons in response to noxious stimuli. Once released the tachykinin binds the neurokinin-1 (NK-1) receptor, giving rise to a range of biological effects including transmission of nociceptive information via second-order neurons in the spinal cord. Evidence from earlier studies suggest that following peripheral nerve damage, spinal reorganization and phenotypic changes in neuronal peptide content could permit an innocuous stimulus to instigate the inappropriate release of substance P and subsequent NK-1 receptor activation. If so, the binding of substance P to the NK-1 receptor may be in part responsible for the abnormal pain perception in peripheral neuropathies. However, this possibility has not been investigated *in vivo*.

In order to further examine this possibility we determined if activation of NK-1 receptors could be induced by an innocuous peripheral stimulus in an animal model of neuropathic pain. Furthermore, we assessed the effect of inhibiting the substance P/NK-1 receptor

interaction in order to justify this approach as an appropriate treatment option for neuropathic pain.

LITERATURE REVIEW

I. ANIMAL MODELS OF NEUROPATHIC PAIN

Research into the mechanisms behind neuropathic pain as well as the discovery of novel analgesics has been greatly facilitated in recent years by the development of a number of reliable animal models. In 1988, Bennett and Xie developed the first animal model of peripheral neuropathic pain known as the chronic constriction injury (CCI) model in the rat (Bennett and Xie, 1988). The model involves loose ligation of the sciatic nerve at mid-thigh level with four chromic gut sutures (Bennett and Xie, 1988). Characteristically, there is an inflammatory reaction in response to the catgut and a consequent loss of most A and some C fibers, but not cell bodies. The model mimics human neuropathic pains as the animal develops spontaneous pain, allodynia and hyperalgesia in the ipsilateral paw. However, there is a large degree of variability between the individual sutures on a given animal, different animals and even more so between examiners. Two years following this first model the partial spinal nerve ligation model (PNL) was developed. This model involves the tight ligation of 33-50% of the sciatic nerve at mid-thigh level, leaving the rest of the nerve untouched (Seltzer et al., 1990). The animal's development of spontaneous pain, allodynia and hyperalgesia is very similar to the Bennett and Xie model. While the PNL model involves less of an inflammatory component than the CCI model, there remains significant variability in the number of neurons ligated per animal, and furthermore, the nerve damage cannot be related to a specific dorsal root ganglion (DRG) as the sciatic nerve divides into to the L4, L5 and L6 spinal nerves in the rat. In 1992, four years after the first animal model of

neuropathic pain was developed, a third novel model known as the spinal nerve ligation model (SNL) emerged. This model involves the tight ligation and complete transection of the L5 and L6 lumbar spinal nerves, which contribute to the sciatic nerve (Kim and Chung, 1992). The model is ideal for examining the changes in the DRG, because the damaged nerves can be compared to L4, which remains intact. This model results in a greater mechanical and thermal allodynia than the two earlier models, however, the injury is central rather than peripheral thus creating a different type of nerve injury (Kim and Chung, 1992). In 1996, the Mosconi and Kruger model was developed. It is a variation of the CCI model, which aims to eliminate the inter-animal inconsistency due to manual tightening of sutures around the sciatic nerve (Mosconi and Kruger, 1996). This peripheral injury model places a 2mm section of split polyethylene tubing around the sciatic nerve of a rat causing dysesthesia, allodynia and hyperalgesia. The model causes three trademark effects. (1) A mechanical allodynia as exhibited by a significant decrease in withdrawal threshold to the application of von Frey hairs; (2) A characteristic guarding of the ipsilateral paw; and (3) A spontaneous lifting of the ipsilateral paw indicative of spontaneous pain (Mosconi and Kruger, 1996; Pitcher et al., 1999). Other recently developed models, which are rarely used to date, include a model involving a highly reproducible photochemically induced ischaemia of the sciatic nerve (Kupers et al., 1998), and one involving tight ligation and lesion of the tibial and common peroneal nerves (Decosterd and Woolf, 2000). Although the majority of animal research has focused on the Bennett and Xie model allowing the changes resulting from the neuropathic surgery to be well defined in this model, the studies reported here employ the Mosconi and Kruger model as we feel it does not incite a significant immune reaction and provides more consistent sensory changes.

II. Elucidated Mechanisms of Neuropathic Pain

The emergence of these reliable and reproducible animal models of neuropathic pain promoted the elucidation of some of the mechanisms behind this pain. There are peripheral, central, and supra-spinal changes that have been identified in the various animal models, which are in some cases backed by evidence in humans. Given the complexity and variability of the painful pathologies, the mechanisms reported here most likely act concurrently to establish and preserve neuropathic pain.

i. Peripheral Changes--Ectopic Discharges

Following nerve injury significant increases in aberrant, spontaneous neuronal firings known as ectopic discharges have been demonstrated in humans experiencing neuropathic pain (Nordin et al., 1984) as well as in a number of animal models, including the CCI model (Kajander et al., 1992). Sub-threshold membrane oscillations were shown to take place in A- and C- fibers and the occurrence of oscillations increased from 10% to 23% of in A-fibers neurons after SNL (Amir et al., 1999). The increase in membrane oscillations would lead to an increased number of ectopic discharges in an individual nerve as well as surrounding nerves due to chemically mediated cross-excitation (Amir and Devor, 1996). Additionally, ectopic discharges may occur in both the injured neurons and intact neighboring neurons. (Yoon et al., 1996; Li et al., 2000). The spontaneous activity is mediated through sodium channels (Matzner and Devor, 1994), and may be the result of changes in the expression of the various sodium ion channel subtypes following peripheral nerve injury (Waxman et al., 1999). Extent of ectopic discharges have been well correlated with severity of pain behaviors in rat models of neuropathic pain, indicating that the spontaneous excitability of primary afferent neurons may be important

in the maintenance of neuropathic pain through spontaneous pain, and contribution to other mechanisms such as central sensitization (Chul Han et al., 2000).

ii. Peripheral Changes--Changes in Sensory Fiber Peptide Expression

Long lasting changes in the expression of peptides and their receptors has been shown to occur following peripheral nerve injury and has been associated with an adaptive response to facilitate recovery and regeneration of damaged neurons (Hokfelt et al., 1994). Conversely, others have suggested the changes may bring about central sensitization and the precipitation of chronic pain states (Coderre and Katz, 1997). Large diameter touch-sensitive Aβ-fibers may change their phenotype to begin to express pain mediators such as substance P (Neumann et al., 1996). Moreover, Aβ-fibers isolated from neuropathic animals two weeks following onset of neuropathy, can be electrically stimulated to release substance P (Malcangio et al., 2000). Results of electrophysiological experiments with spinalized naïve and neuropathic rats demonstrated that the afterdischarge in the dorsal horn in response to a noxious pinch stimulus was greater in magnitude and duration in neuropathic rats while the initial discharge remained unchanged (Pitcher and Henry, 2000). This result is best explained by the phenotypic change of myelinated fibers to express substance P leading to the prolonged afterdischarge. In fact, a similar afterdischarge was recreated in electrophysiological experiments using neuropathic animals given an innocuous stimulation, which was blocked with an NK-1 receptor antagonist (Pitcher et al., in print). In all, these studies implicate the release of substance P from Aβ-fibers in peripherally nerve-injured animals.

iii. Central Changes--Spinal Reorganization

Centrally, a considerable degree of reorganization of afferent fibers innervating the spinal dorsal horn has been reported. In normal animals, nociceptive myelinated Aδ-fibers and

unmyelinated C-fibers generally project into laminas I and II, while myelinated Aβ-fibers terminate in the deeper laminas III and IV of the dorsal horn. However, following peripheral nerve injury, remission of C-fibers innervating lamina II and subsequent sprouting of Aβ-fibers into the vacancy has been reported (Woolf et al., 1992; Koerber et al., 1994; Lekan et al., 1996). Allowing the proper chemical mediators, this could result in low-threshold mechanical stimulation of Aβ-fibers in the periphery being relayed as painful stimuli by second order nociceptive spinal neurons contacted in lamina II, and could explain the allodynia observed in neuropathic pain models. However, the earliest spinal reorganization was demonstrated at one week after sciatic nerve axonomy (Woolf et al., 1992), despite the appearance of allodynia as early as three days in most animal models. Regardless of this time discrepancy, spinal reorganization remains a major mechanistic theory of neuropathic pain.

iv. Central Changes--Central Sensitization

The augmentation of dorsal horn neuron responses to normal afferent input, such as a decrease in activation threshold or a sustained state of spinal hyperexcitability, is known as central sensitization. This phenomenon is thought to occur in many chronic pain states, including neuropathic pains. Evidence to date indicates that activation of the ionotropic N-methyl-D-asperatate (NMDA) glutamate receptor subtype may be essential to the process of central sensitization in both inflammatory and nerve-injury induced pain. However, other molecules including the tachykinin substance P have been implicated as playing a role in central sensitization (Urban et al., 1994). For glutamate to access its NMDA receptor the removal of a magnesium-dependant channel block and subsequent receptor phosphorylation are necessary. Once the receptor is activated, the secondary neuron would be hypersensitive to the release of glutamate by primary afferents. This

effect may be further enhanced by the release of brain-derived neurotrophic factor from unmyelinated primary afferents, which binds its receptor, trkB (Thompson et al., 1999). The importance of the NMDA receptor is compounded by the fact that glutamate concentration in the dorsal horn increases in neuropathic animals (Kawamata and Omote, 1996), and that hyperalgesia can be prevented by the administration of an NMDA receptor antagonist in the CCI model (Davar et al., 1991; Mao et al., 1992; Sotgiu and Biella, 2000). A central sensitization could create a state of spinal hyperexcitability leading to neuropathic pains.

v. Central Changes--Depression of inhibitory pathways

The primary inhibitory pathway of the central nervous system is the γ-aminobutyric (GABA) pathway. GABA gates the entrance of chloride ions into nerve cells, and can thus block the presynaptic release of neurotransmitters. It has been hypothesized that the loss or depression of this inhibitory action could explain the perceived increase in spinal excitability (Sivilotti and Woolf, 1994). Intrathecal administration of both $GABA_A$ and $GABA_B$ receptor agonists can attenuate allodynia in the spinal nerve ligation model of neuropathic pain (Hwang and Yaksh, 1997). Furthermore, a significant decrease in the concentration of extracellular GABA has been reported in nerve injured rats (Stiller et al., 1996) as well as a significant decrease in the profile of GABAergic interneurons in laminas I-III as early as three days following CCI surgery (Ibuki et al., 1997). Also, hyperchromatic "dark neurons" are consistently found in the dorsal horn following CCI, which are indicative of trans-synaptic degradation, possibly GABAergic interneurons (Sugimoto et al., 1990; Hama et al., 1994). The effect of a loss of inhibitory interneurons following peripheral nerve injury most likely plays a role in the development and maintenance of neuropathic pains.

vi. Supra-spinal Changes

Reorganization and alteration in neuronal responsiveness may also occur in many supraspinal centers. Although data in this area remains limited, there is some evidence that changes may occur at levels of the cortex and thalamic nuclei. The mechanical allodynia observed in SNL rats, was reported abolished by complete spinal transection at the mid-thoracic level as evaluated using von Frey hair filaments (Bian et al., 1998). Furthermore, hemisection of the spinal cord at T8 ipsilateral, but not contralateral to the SNL injury blocked the mechanical allodynia (Sung et al., 1998). Of note, however, is that the spinal transections had no effect on thermal hyperalgesia. The effects on mechanical allodynia were deemed exclusive of paralysis of the animals, as spinal nociceptive response from a pinch stimulus persisted (Bian et al., 1998). This evidence does not discount the importance of persistent peripheral drive and other peripheral and central changes, however, it demonstrates the fact that higher brain centers play a role in transmitting and modulating the pain responses observed behaviorally in neuropathic animals.

The many distinct changes that occur peripherally, centrally and supraspinally are most likely not independent of one another. They almost certainly cause and affect each other and all play a significant role in the development and maintenance of chronic pains. In the case of neuropathic pain, there is likely no single distinct trigger rather, numerous changes occur throughout the entire nervous system in consequence of the initial peripheral injury.

III. Substance P and the NK-1 Receptor

Substance P is an 11 amino acid peptide of the tachykinin family, which has diverse physiologic effects in the central nervous system and in peripheral tissues. Under normal conditions, the neurotransmitter is strictly found within the small caliber Aδ- and C-fiber subpopulations of primary afferent neurons (Hokfelt et al., 1975; Cuello and Kanazawa, 1978). As a result it is only released following a noxious thermal, mechanical or chemical stimulus (Duggan and Hendry, 1986; Brodin et al., 1987; Duggan et al., 1988; McCarson and Goldstein, 1991). Although it is just one of the many peptides released into the superficial dorsal horn substance P has been shown to play a pivotal role in the increased excitability of nociceptive pathways (Henry, 1976). Moreover, intrathecal administration of substance P causes hyperalgesia in the tail-flick test (Yashpal et al., 1982).

Substance P has long been considered the preferred ligand of the neurokinin-1 (NK-1) receptor. The interaction of substance P and NK-1 receptor has been shown to have distinct physiological effects. Iontophoretic application of substance P on the dorsal horn activates NK-1 receptors on second order nociceptive neurons, which elicit slow excitatory post-synaptic potentials (De Koninck and Henry, 1991; Radhakrishnan and Henry, 1991). Furthermore, hyperalgesia in the tail-flick test as a result of intrathecal substance P is known to be due to activation of NK-1 receptors as it is alleviated by the antagonist CP-96,345 but not its stereoisomer CP-96,344 (Yashpal et al., 1993).

IV. Substance P and the NK-1 Receptor in Neuropathic Pain

In conditions of peripheral inflammation or peripheral nerve damage the peptide content of nerves and membrane receptors expressed may change significantly (Hokfelt et al., 1994). Following nerve injury Aβ-fibers may begin to produce substance P (Neumann et al., 1996), and sprout into lamina II (Koerber et al., 1994) where secondary neurons express NK-1 nociceptors. Presumably resulting from its expression in Aβ-fibers, an upregulation preprotachykinin-A mRNA, the precursor to substance P, has been shown in the dorsal root ganglion of CCI rats (Marchand et al., 1994; Noguchi et al., 1994). In addition, substance P mRNA was shown to increase in the dorsal horn ipsilateral to the partial sciatic nerve ligation, however, there was a return to baseline one week later (Delander et al., 1997). Conversely, earlier studies demonstrate decreased substance P immunoreactivitity in the dorsal horns of neuropathic animals (Cameron et al., 1991; Garrison et al., 1993; Munglani et al., 1995). Notably, an upregulation of NK-1 receptors in the ipsilateral dorsal horn has been reported and this may provide an increased sensitivity to the release of substance P (Aanonsen et al., 1992; Goff et al., 1998).

Preclinical studies have implicated the NK-1 receptor a principal mediator of certain chronic pain states, including neuropathic pain. Cumberbatch and colleagues demonstrated a reversal of mechanical allodynia in the CCI model of neuropathic pain following i.v. administration of the NK-1 receptor antagonist GR205171, using an innocuous paw pressure test (Cumberbatch et al., 1998). Furthermore, Coudore-Civiale and coworkers showed that in the CCI model, intrathecally administered NK-1 antagonists (CP-96,345, RP-67,580, and SR-140,333) raised the pressure required from the Randall-Selitto apparatus to instigate vocalization of rats (Coudore-Civiale et al., 1998). Campbell et al. demonstrated that NK-1 receptor antagonists (SDZ NKT and LY

303,870) significantly decreased mechanical hyperalgesia as measured by paw pressure test in a guinea-pig model of neuropathic pain (Campbell et al., 1998). Additionally, Gonzalez et al. showed the NK-1 receptor antagonist CI-1021 to dose dependently inhibit static mechanical allodynia assessed by von Frey hairs in the CCI model (Gonzalez et al., 2000). Moreover, Mansikka and colleagues showed that unlike controls, transgenic mice lacking the NK-1 receptor do not exhibit increased withdrawal frequencies to von Frey hairs indicative of mechanical allodynia, following L5 spinal ligation (Mansikka et al., 2000). Finally, Cahill and Coderre showed that pre- or post-surgery treatment of neuropathic rats with an intrathecally administered NK-1 antagonist (L-732,138) attenuates hyperalgesia (Cahill and Coderre., 2002). In contrast, the antagonist RP 97530 was shown to be ineffective in treating diabetic neuropathy in rats (Malcangio and Tomlinson, 1998). Also, mechanical allodynia in substance P/neurokinin-A knockout mice rendered neuropathic did not differ significantly from wild-type (Cao et al., 1988). It is not unlikely that these differences are merely due to methodological discrepancies, or differences in the models employed.

Despite the reported success of NK-1 receptor antagonists in animal models, the role of the NK-1 receptor in human neuropathic pains has been questioned due to the lack of analgesia in clinical trials (Suarez et al., 1994; Goldstein and Wang, 1999; Block et al., 1998; Boyce and Hill, 2000). However, the failure of the NK-1 receptor antagonists in human trials is currently debated due to the recent awareness of species differences as well as lack of appropriate parameters such as the type of pain studied (Hill, 2000; Urban and Fox, 2000; Laird, 2001). Further studies are necessary to assess the possible analgesic effects of an NK-1 receptor antagonist in humans.

NK-1 RECEPTORS ARE ACTIVATED FOLLOWING AN INNOCUOUS PERIPHERAL STIMULATION CONTRIBUTING TO MECHANICAL ALLODYNIA IN AN ANIMAL MODEL OF NEUROPATHIC PAIN

The material contained in this manuscript is to be submitted to the Journal of Neuroscience. The discussion section of the manuscript has been expanded to avoid repetition in the thesis discussion.

I. ABSTRACT

The tachykinin substance P and its neurokinin-1 (NK-1) receptor are implicated in the mechanical allodynia of neuropathic pain. Despite demonstrated phenotypic changes in the peptide content of primary afferents and clear spinal reorganization, the impact on substance P/NK-1 interaction has not been examined *in vivo*. Here, we examine whether a normally innocuous peripheral stimulus is capable of eliciting activation of NK-1 receptors in rats rendered neuropathic.

We determined that the high intensity stimulus requisite for release of substance P and activation of NK-1 receptors in normal animals was not required to incite a similar effect in neuropathic animals. Indeed, a normally innocuous tactile stimulation of the affected area was sufficient to cause heterosegmental inhibition in the tail-flick test, and increased plasma extravasation in the ipsilateral paw; effects which were reversed by pretreatment with the NK-1 receptor antagonist CP-96,345.

We subsequently investigated and compared CP-96,345, morphine gabapentin, phenytoin, verapamil, diltiazem, methylene blue, and a functional NK-1 receptor antisense oligonucleotide for efficacy in alleviating the characteristic mechanical allodynia. We found that CP-96,345 and the antisense oligonucleotide were far more effective than morphine, while other drugs were without significant effect. Finally, we used mass spectrometry to quantify substance P in the spinal cord of neuropathic and naïve animals and found significant upregulation following cuff implantation. We conclude that a normally innocuous stimulus could activate NK-1 receptors, presumably by release of substance P, and that this activation may be responsible for mechanical allodynia in conditions of peripheral neuropathy.

II. INTRODUCTION

Neuropathic pain is a chronic, often debilitating condition defined as pain initiated or caused by a primary lesion or dysfunction in the nervous system. Affected individuals often complain of constant burning or stabbing pains, which are associated with allodynia (painful sensation of a normally innocuous stimulus), hyperalgesia (increased painful sensation of an already painful stimulus) and dysesthesia (spontaneous pain). Neuropathic pain is reported most often as neuropathic lower back pain, diabetic neuropathy, postherpetic neuralgia, and reflex sympathetic dystrophy (Bennett, 1998). Traditional analgesics such as morphine and ibuprofen are generally ineffective against neuropathic pain at acceptable doses (Arnér and Meyerson, 1988; Max et al., 1988; Kupers et al., 1991). Furthermore, aside from varied patient tolerance, currently employed non-traditional analgesics including gabapentin and amytriptyline seem to be miraculously effective or practically useless (McQuay et al., 1996; Sindrup and Jensen, 1999). The reason for the dramatic discrepancy in patient response to the available drugs is unknown, and elucidation of the mechanistic basis of neuropathic pain is necessary to rationalize this difference and discover novel analgesics for the many patients who represent an area of unmet therapeutic need.

The emergence of a number of reliable animal models since the original chronic constriction injury model (Bennett and Xie, 1988) has promoted great advancement of our knowledge of the possible mechanisms responsible for neuropathic pain, and provided a vehicle to test novel analgesics. Central, peripheral and cerebral mechanisms all seem to influence the aberrant pain sensations. Phenotypic changes in nerve fibers have been reported following peripheral nerve injury, including the production of pain mediators such as substance P, by normally touch transmitting Aβ-fibers (Neumann et al.,

1996). In addition, NK-1 antagonists have been repeatedly demonstrated to alleviate mechanical allodynia in animal models of neuropathic pain (Cumberbatch et al., 1998; Coudore-Civiale et al., 1998; Campbell et al., 1998; Gonzalez et al., 2000; Cahill and Coderre, 2002). Furthermore, spinal reorganization has been implicated in being a part of the neuropathic mechanism. Specifically, the remission of C-fibers from lamina II of the dorsal horn and subsequent growth of A-beta fibers into the vacated space has been demonstrated (Woolf et al., 1992; Koerber er al., 1994; Lekan et al., 1996). Ectopic discharges, central sensitization and depression of inhibitory spinal pathways have also been implicated as possible mechanisms of neuropathic pain.

In this study we further examine the role of substance P and the NK-1 receptor in the Mosconi and Kruger rat model of neuropathic pain (Mosconi and Kruger, 1996). We demonstrate that following an innocuous stimulation there is activation of the NK-1 receptor in neuropathic, but not naïve animals. We demonstrate this phenomenon indirectly through both tail-flick testing and peripheral plasma extravasation. Presumably this activation is due to the release of substance P, since it is involved in excitation of nociceptive pathways (Henry, 1976) at the NK-1 receptor. Therefore, we examine the effect of an NK-1 receptor antagonist on mechanical allodynia of our neuropathic animals. Discovering a strong analgesic effect, far greater that the other currently employed analgesics tested we designed a functional antisense oligonucleotide against the NK-1 receptor, to confirm the result of the antagonist. The treatment alleviated the mechanical allodynia in neuropathic animals in a manner very similar to the NK-1 receptor antagonist. Finally, to substance P, and implicate this tachykinin in the activation of NK-1 receptor, we conducted a mass spectrometric analysis of lumbar spinal cord. We found an upregulation of substance P in neuropathic animals as compared to

naive. Taken together, these data demonstrate that in neuropathic animals NK-1 receptors are activated following an innocuous stimulus having effects centrally and peripherally, probably due to the release of substance P. Moreover when this activation of the NK-1 receptor is blocked, the mechanical allodynia experienced by neuropathic animals is greatly alleviated.

III. MATERIALS AND METHODS

i. Animals

Experiments were performed on male Sprague-Dawley rats (300-350 g; Charles River, Quebec, Canada) housed in groups of two per cage. Rats were maintained on a 12/12 hour light/dark cycle and were allowed free access to food and water. Experiments were carried out according to a protocol approved by the animal care committee at McGill University and in accordance with guidelines from the Canadian Council on Animal Care and I.A.S.P. Committee for Research and Ethical Issues. All experiments were performed during the light cycle.

ii. Surgical Procedures

a. Sciatic nerve constriction

Sciatic nerve injury was accomplished by the method previously described by Mosconi and Kruger (Mosconi and Kruger, 1996). Briefly, rats were anaesthetized with ketamine/xylazine and the lateral left thigh was shaved and swabbed with 70% ethanol. A skin incision was made in the cleaned area. The sciatic nerve was exposed by blunt dissection and gently freed from adhering tissue. A polyethylene (PE-90) cuff (2 mm in length) was wrapped around the entire sciatic nerve. Care was taken to ensure the cuff did not pinch the nerve and that it was not too tight so to occlude the perineural blood flow. The separated muscle was stitched and the incision was closed with wound clips. Animals received topical antibiotic (Furasone O), intra-muscular antibiotic (Tribrissen 24%) and subcutaneous saline (5 ml).

b. Intrathecal Surgery

Each rat was implanted with a chronic indwelling intrathecal catheter (PE-10) under ketamine/xylazine/acepromazine anaesthesia (4.88/0.49/0.06 mg/100 g, i.m.). The

catheter was inserted through an incision in the dura mater at the atlanto-occipital junction and was positioned so that the inner tip lay at the lower thoracic vertebral level (spinal cord L3-L5). Spinous processes were used as landmarks for this positioning. The other end of the catheter was anchored in place using dental cement to fix it to a screw embedded in the skull. The exact location of the inner tip was confirmed for all animals by performing a lamenectomy post mortem. In addition, the viability of the intrathecal catheter was determined by loss of sensation to hind paws following a 10 µl injection of lidocaine (1%) within two minutes of administration. The rats were allowed to recover from surgery for 7 to 10 days prior to attaching an alzet minipump for oligonucleotide delivery.

iii. Drugs

All drugs were dissolved in physiological saline. The NK-1 receptor antagonist CP-96,345 was administered in a 5mg/kg intraperitoneal (i.p.) dose 20 minutes before von Frey hair testing of mechanical allodynia. Morphine was administered in a 5mg/kg i.p. dose 20 minutes prior to von Frey testing. Gabapentin was administered in a 50mg/kg i.p. dose 45 minutes prior to von Frey testing. Phenytoin was administered via an intrathecal (i.th.) catheter in a 36.5nmol dose prior to von Frey testing. 30ug methylene blue was administered i.th. prior to von Frey testing. 50ug verapamil was administered i.th. prior to von Frey testing. 100ug diltiazem was administered prior to von Frey testing. Control for all drugs was the vehicle, saline.

iv. Oligonucleotides

The 21-base antisense oligodeoxynucleotide with natural unmodified phosphodiester backbone was designed according to the primary sequence of the rat NK-1 cDNA

(Hershey et al., 1991). Natural ODNs were chosen due to the lower propensity to produce toxicity and limited non-selective protein binding as compared to phosphorothioate backbone derivatives (Akhtar & Agrawal, 1997; Wahlestedt, 1994). The sequence of the NK-1 antisense probe was: 5'-GACGTTATCCATTTTGGGGCA-3', targeted around the translation initiation site (nucleotides 576 to 578). The mismatch probe was: 5'-GAGCTTTACTATTGTGGG-3'. The mismatch control oligonucleotide probe showed neither internal complementarities nor resemblance to any other known sequences according to the GenBank Database. Custom synthesis and HPLC-purification was performed by Keystone Labs (Camarillo, CA). ODNs were dissolved in artificial cerebrospinal fluid (mM: NaCl 128.6, KCl 2.6, $MgCl_2$ 1.0, $CaCl_2$ 1.4, pH 7.4; ACSF), to give 75 μg/10 μl final concentration for infusion. The dose was chosen based on effective concentrations previously reported (Wahlestedt, 1994).

For continuous infusion into the intrathecal lumbar spinal cord region, each rat was implanted with an osmotic minipump [Alza Minipump, model 2001 (Palo Alto, CA)] that was attached to the previously implanted cannula. The pump and cannula were filled with one of the above solutions, or ACSF control, under sterile conditions, and then the cannula was joined to the pump, avoiding air bubble formation. The position of the tip of the cannula with respect to the level of spinal segment was ascertained at the end of each experiment, and only data from animals with correct cannula placement (L3–L6) were used in analysis. No animal showing abnormal gait or paralysis during the 21-day period was included in the study.

v. Behavioral experiments

a. Mechanical withdrawal threshold

Mechanical response thresholds were determined by measuring the hind paw withdrawal response to von Frey filament stimulation according to the method described by Pitcher (Pitcher et al., 1999). In brief, animals were placed in a Plexiglas® box (30 x 30 x 30 cm) with an opaque plastic floor (3 mm thick with 1.5 mm diameter holes in perpendicular holes 5 mm apart) through which the von Frey filaments (Stoelting) were applied to the soft tissue of the plantar surface of the injured hind paw. Filaments were applied in ascending order, beginning at 0.25 g, to determine the filament closest to the threshold of response. Each filament was applied to the hind paw five times for two sec at 10-second intervals. A withdrawal response was considered if the hind paw was completely removed from the surface of the platform. The withdrawal response was recorded for the filament that successfully stimulated withdrawal 4/5 or 5/5 times. The gram force of each hair was determined at the end of each testing period.

b. Tail flick test

Each rat was placed in a plastic restrainer covered with a black cloth and a projector bulb was focused on the tail 4 cm form the tip. Response latency was recorded when the rat flicked its tail from the light beam which was determined by a connection to a photodetector that in turn was connected to a timer that measured to 0.01 seconds (Isabel et al., 1981). Reaction time was measured at three-minute intervals. The intensity of the stimulus was set to give baseline reaction times of eight to twelve seconds.

Response to an innocuous peripheral stimulation

In order to determine the effect of an innocuous peripheral stimulus on tail-flick latency in neuropathic and naïve rats, eight animals were conditioned to the restrainer and testing

regimen. All testing days were done using the following procedure. (1) The rat was placed in restrainer and first tail-flick latency reading taken after 3 minutes. (2) Baseline readings were taken until five consecutive consistent readings resulted, the average of which was considered the baseline response. (3) The rat was removed from the restrainer and wrapped in a towel with only the left hind paw exposed, and held in this position for 30 seconds as a control handling. (4) The rat was returned to the restrainer and three more readings were taken. (5) The rat was removed from the restrainer and held in the same wrapped position as above, however, the exposed paw was an innocuous tactile stimulation of the plantar surface was applied for 30 seconds with a fine brush. (6) The rat was returned to the restrainer and five final readings were taken. Three days of baseline readings were taken as well as withdrawal thresholds using von Frey hairs prior to cuff implantation on the left sciatic nerve. Five days after cuff implantation, the animals were confirmed neuropathic by demonstrating mechanical allodynia in von Frey hair test. Six and seven days after cuff implantation, animals were reevaluated in the above tail-flick testing procedure. Eight and nine days after cuff implantation the NK-1 antagonist CP-96,345 was administered one minute prior to testing. For removal from the restrainer, the tail of each animal was held to a sliding plastic floor added to the apparatus and pulled-out in one motion as a single unit to guarantee there was no erroneous movement or stimulation of the hind paws. All reaction times were calculated as a percentage of the baseline value calculated for each day. Results are expressed as the average for all rats over all days for each particular treatment.

Response to intrathecal substance P before and after oligonucleotide treatment

Three baseline latencies were recorded prior to intrathecal injection of substance P (6.5 nmol/10 μl, Peninsula lab) in ACSF. This was followed by 10 μl of ACSF to flush the

catheter (approximate internal volume 8 µl). The intrathecal injection was timed to end 1 min prior to the next latency test. Three more readings were taken after the intrathecal injection. The mean of three baseline readings immediately prior to substance P administration was taken as 100% response for each rat. All subsequent reaction times for each rat were expressed as a percentage of this mean baseline reaction time.

vi. Plasma extravasation experiments

Animals were anaesthetized with sodium pentobarbital at a dosage of 50mg/kg. The tail vein of the animals was injected with 62.5mg/kg of Evan's Blue dye (Sigma) dissolved 25mg/ml in physiological saline. Thirty minutes following Evan's Blue injection animals were perfused with 500ml physiological saline. Subsequently the paws were removed immediately above the ankle joint, weighed and placed in vial with 15ml of formamide overnight in an oven at 60^0C. Twenty-four hours later, the fluid in the vials was filtered and evaluated for Evan's Blue content by its absorbance measure by colour spectrophotometer compared to pure formamide at wavelength 600nm. Results were calculated according to the following formula:

[ipsilateral absorbance / ipsilateral weight] / [contralateral absorbance / contralateral weight]

Six groups were examined under this procedure. (1) Naïve animals. (2) Stimulated naïve animals. (3) Neuropathic animals. (4) Stimulated neuropathic animals. (5) Stimulated neuropathic animals pre-treated with saline 25 minutes prior to the brush stimulus. (6) Stimulated neuropathic animals pre-treated with CP-96, 345 25 minutes prior to the brush stimulus. The stimulus consisted of a one-minute innocuous tactile stimulation of the plantar surface ipsilateral hind paw with a fine brush.

vii. Western blotting experiments

For preparation of tissue membranes, rats were killed by decapitation and spinal cords were quickly removed by spinal ejection and placed on ice for removal of the dura mater. Dorsal lumbar regions were homogenized with a Polytron in 50 mM Trisma base, pH 7.0 and 4 mM EDTA with protease inhibitors (Complete™ Protease inhibitor tablets, Roche Molecular Biochemicals, Laval, QC). Samples were centrifuged at 4°C for 10 minutes at 1,000 rpm (Sorval RC5C Plus). The supernatant was collected and the pellet re-suspended in buffer "A" and centrifuged again at 4°C for 10 minutes. The supernatant from the second spin was combined with that of the first for each sample and centrifuged at 4°C for 10 minutes at 46,000 rpm (Sorval Discovery 90). The pellets were then re-suspended in buffer consisting of 50 mM Trisma base, pH 7.0 and 0.2 mM EDTA with protease inhibitors by vortexing and brief sonication (2 seconds).

Spinal cord membranes were denatured using 6X Laemmli sample buffer (0.375 mM Trisma base, pH 6.8, 12% w/v SDS, 30% v/v glycerol, 12% v/v β-mercaptoethanol, 0.2% w/v bromophenol blue). Samples were resolved using 10% Tris-glycine pre-cast gels (Novex, San Diego, CA) and the proteins were electroblotted onto nitrocellulose membranes. A pre-stained kaleidoscope molecular mass marker from BioRad Laboratories (Richmond, CA) and/or a biotinylated molecular weight marker from New England Biolabs (Mississauga, ON) were used to calibrate the gels. Nitrocellulose membranes were incubated with 1% bovine serum albumin (BSA) and 1% chicken ovalbumin (OA) in 25 mM Tris with 150 mM sodium chloride (TBS) containing 0.05% Tween 20 (TBS+T) overnight at 4°C to block non-specific sites. Nitrocellulose membranes were then incubated for overnight at 4°C with NK-1 antisera (1:1000,

generous gift from Dr Krause) in TBS+T with 1% BSA and 1% OA. Specificity of the antisera has previously been characterized (Ribeiro-Da-Silva et al., 2000). Bound antibodies and the biotinylated molecular weight marker were visualized using an HRP-conjugated goat anti-rabbit secondary antibody (Amersham Pharmacia Biotech, Inc., Baie D'Urfé, QC) diluted 1:4000 and an HRP-conjugated anti-biotin antibody diluted 1:10,000 (New England Biolabs), respectively, in TBS+T and 5% milk powder (Carnation, Don Mills, ON) followed by chemiluminescence reagents (NEN Life Science Products, Boston, MA). Blots were digitized by scanning with an Agfa Duoscan T1200 and image processing was performed using Photoshop version 4.0.1 (Adobe Systems Inc., San Jose, CA) on an IBM-compatible computer.

viii. Immunohistochemistry

Rats (n=3 per group) were anaesthetised with sodium pentobarbital (70 mg/kg, intraperitoneal) and perfused through the aortic arch with a freshly prepared solution of 4% PFA in 0.1 M PB (500 ml, pH 7.4) at 4°C. Spinal cords were rapidly removed by hydrostatic propulsion and tissue was then post-fixed in the same fixative solution by immersion for one hour followed by cryoprotection in 30% sucrose in 0.2 M PB, both at 4°C. Transverse sections (50 μm) were cut on a freezing microtome and collected in 0.1 M phosphate buffered saline containing 0.2% Triton X-100, pH 7.4 (PBST). Free-floating sections were washed and then incubated with 1% sodium borohydride. Sections were copiously washed prior to incubation for one hour in a blocking solution containing 10% normal goat serum and 10% normal horse serum (NHS) at room temperature for two hours. Sections were then incubated at 4°C for 48 hours with a polyclonal antibody raised in rabbit against the NK-1 receptor (gift from Dr. J. Krause) diluted to 1/100 in

PBST and rat antibody against substance P (Medicorp, Canada, Suresh et al., 1986) diluted to 1/10 containing 5% NHS. After rinsing in PBST, the sections were incubated at room temperature for 2 h in biotinylated goat anti-rabbit IgG (Vector laboratories, Burlingame, CA) diluted 1:200 in PBST. Sections were thoroughly washed with PBST and incubated in rhodamine X-conjugated donkey anti-rat antibody (Jackson immunoresearch laboratories, West Grove, PA) diluted to 1:50 and streptavidine-Alexa 488™ (Molecular probes, Eugene, OR) diluted to 1:150 for two hours protected from light. Sections were washed and mounted with Aquamount onto gelatin-coated slides. Sections were examined under a Zeiss 510 laser scanning microscope equipped with argon-krypton and helium-neon lasers attached to an Axiovert 100 inverted microscope (Carl Zeiss Canada Ltd., Toronto, ON). Appropriate filter sets for independent detection of Alexa 488 and rhodamine were imposed and identical parameters were used to acquire the images for all conditions. Acquired images were processed using Photoshop version 4.0 or 5.0 (Adobe Systems Inc.) on an IBM compatible computer.

ix. Mass spectrometry

Mass spectrometric analysis was performed by MDS Pharma Services Inc., a division of Phoenix International. Neuropathic and naïve animals were anesthetized with halothane and their lumbar spinal cord removed. The section of cord was cut into 1mm cubes and submerged in extracting solution (0.5ml water, 0.5ml acetonitrile, 10µl trifluoroacetic acid (TFA)). The mixture was sonicated for five minutes, vortexed for two minutes, then centrifuged at 27,000 * g for ten minutes. Supernatant was collected, and then extraction procedure repeated twice more on the remaining pellet. Supernatants were combined and filtered through a 0.45µm filter to remove any remaining particles. Peptides were removed and desalted with ZipTip cartridges (Millipore Corp.) containing C_{18} resin. The

final preparation was concentrated to 20µl in a Speed Vac (Savant) before infusing into a Sciex Pulsari QSTAR mass spectrometer equipped with electrospray ion source. The spectra were manually interpreted with the aid of a peptide database (Matrix Science).

x. Statistical analysis

All data are expressed as means ± S.E.M. Statistical analysis on the time course mechanical withdrawal threshold or tail-flick tests were performed using repeated measures two-way analysis of variance followed by Tukey's Wholly Significant Difference test for *post hoc* comparisons. Stasistical analysis on plasma extravasation experiments was preformed using the unpaired Students *t*-test. Statistical analysis of acute mechanical threshold studies was performed using the paired Students *t*-test. Significance was set at $p<0.05$ for all analyses.

IV. RESULTS

i. Effect of an innocuous peripheral stimulation of the hind paw of neuropathic and naïve animals in the tail-flick latency test

To discover the effect of an innocuous peripheral stimulation on the activation of NK-1 receptors in the spinal cord, we used the tail flick test (for procedure see Methods). Naïve animals consistently demonstrated a slight decrease in tail-flick latency following the control handling and another similar decrease after the innocuous peripheral stimulation of the hind paw (Fig. 1). Five days after cuff implantation the animals were deemed neuropathic as they displayed mechanical allodynia when tested with von Frey hairs (data not shown). Six and seven days after cuff implantation the animals displayed the same response to the control handling. However, following the brush stimulation, the animals exhibited a transient increase in tail-flick latency lasting one to four minutes, and subsequently decreasing latency time to match the naïve animals. On days eight and nine after cuff implantation, the animals were given CP-96,345 one minute prior to testing, about 26 minutes in advance of the brush stimulus. The tail-flick latency decreased slightly following control handling and again following brush stimulation. The animals treated with CP-96,345 did not display the transient increase following the innocuous peripheral stimulation seen in the untreated neuropathic animals. Statistical analysis by two-way analysis of variance and post-hoc Tukey test revealed that the first time point following brush stimulation in untreated neuropathic animals was significantly different from both naïve and CP-96,345 treated neuropathic animals. At any other given time point there were no significant differences between the three groups (naïve, neuropathic, CP-96,345 treated neuropathic).

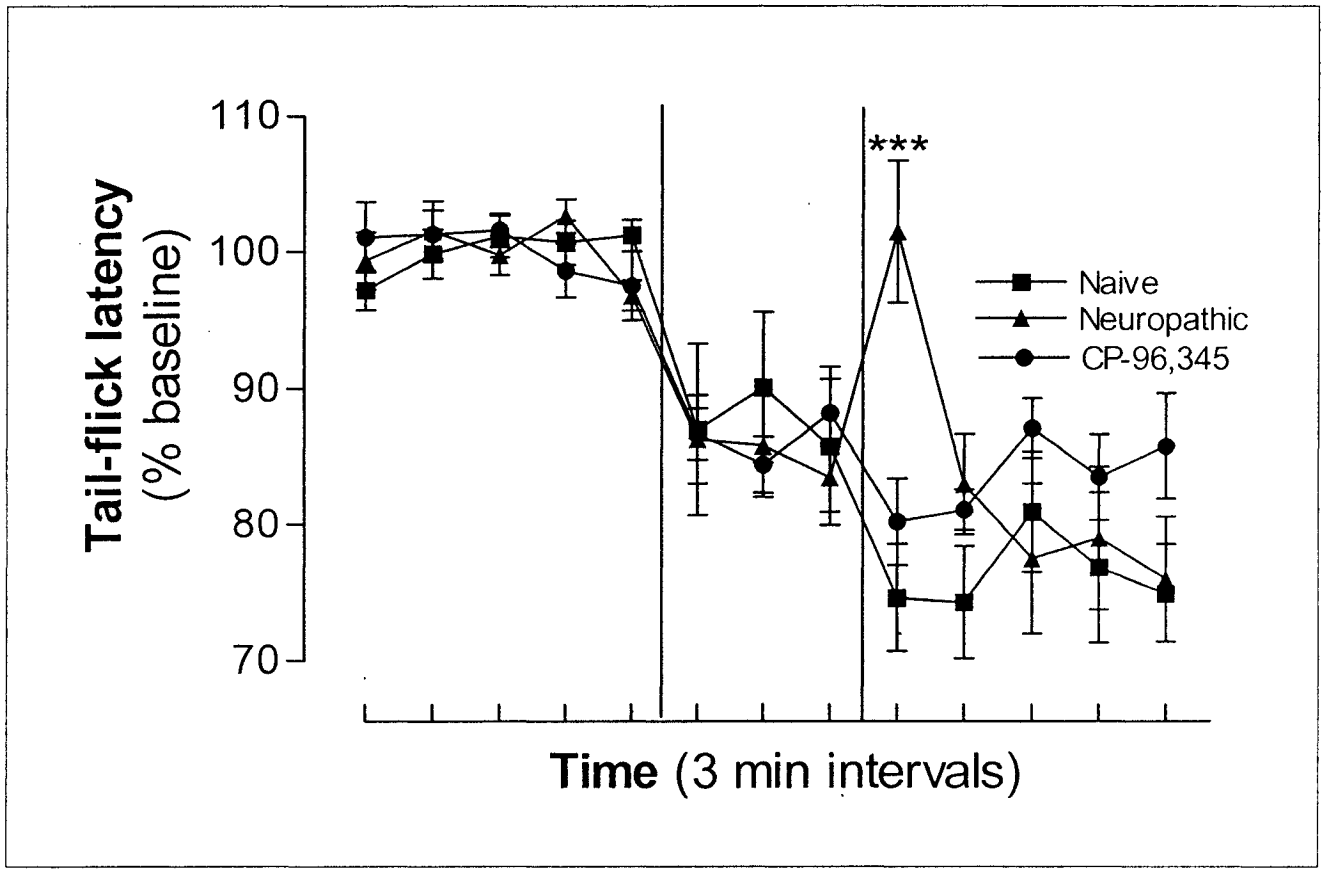

FIGURE 1. NK-1 receptors are activated following an innocuous peripheral stimulation. Animals were trained in a restrainer and handled for one-week prior to start of testing. Animals were placed in the restrainer and tail-flick latency readings taken every three minutes. After five readings, the animals were carefully removed from the restrainer and held for 30 seconds wrapped in a towel with their left paw exposed (indicated by first vertical line). After control handling, animals were returned to the restrainer and three more readings taken. The animals were again removed and held in the same position as the control handling, however, this time their left paw was given a light tactile stimulation for 30 seconds with a paintbrush at 23 min (indicated by second vertical line). Upon return to the restrainer five more readings were taken. Three days of baseline readings were taken. Subsequently a 2mm polyethylene cuff was implanted on the sciatic nerve of the left hind limb. The same procedure as above was performed days six and seven after implantation. Again the same procedure was preformed on days nine and ten after implantation, however, this time CP-96,345 was given 5mg/kg i.p., one minute prior to the first reading. Figure shows the average for all eight animals over all test days. Results are expressed as %baseline. Baseline is established by the average of the first five readings each day. Analysis of the data by two-way ANOVA and post-hoc Tukey test revealed the only significant differences to be in baseline versus post-surgery groups, and in post-surgery versus CP-96,345 treatment groups at time 24 min, *** $p<0.001$ for both comparisons.

ii. Effect of an innocuous peripheral stimulation on plasma extravasation in the hind paws of neuropathic and naïve animals

In order to assess the activation of the NK-1 receptor following an innocuous peripheral stimulation, plasma extravasation was assessed by Evan's Blue dye in the hind paws of naïve and neuropathic animals following saline perfusion. Initially, naïve animals were examined for any effect of an innocuous peripheral stimulation of the left hind paw. No significant difference between unstimulated and stimulated naïve animals was observed (Fig 2). Also, there was no difference in plasma extravasation in the two paws. Animals were cuff implanted and deemed neuropathic by von Frey hair analysis prior to testing (data not shown). Seven days after cuff implantation, plasma extravasation was examined with and without innocuous peripheral stimulation. The piece of plastic pipe in the animal cages was removed as the edges could cause an anomalous stimulation. There was no significant difference between unstimulated neuropathic animals and naïve animals. There was however, a statistically significant increase in plasma extravasation in the ipsilateral paw of stimulated neuropathic animals. This increased plasma extravasation, was significantly attenuated by the NK-1 antagonist CP-96,345, but not by the vehicle, saline. Statistical analysis by unpaired student's t-test indicated a significant difference between saline and CP-96,345 treated, innocuously stimulated neuropathic animals.

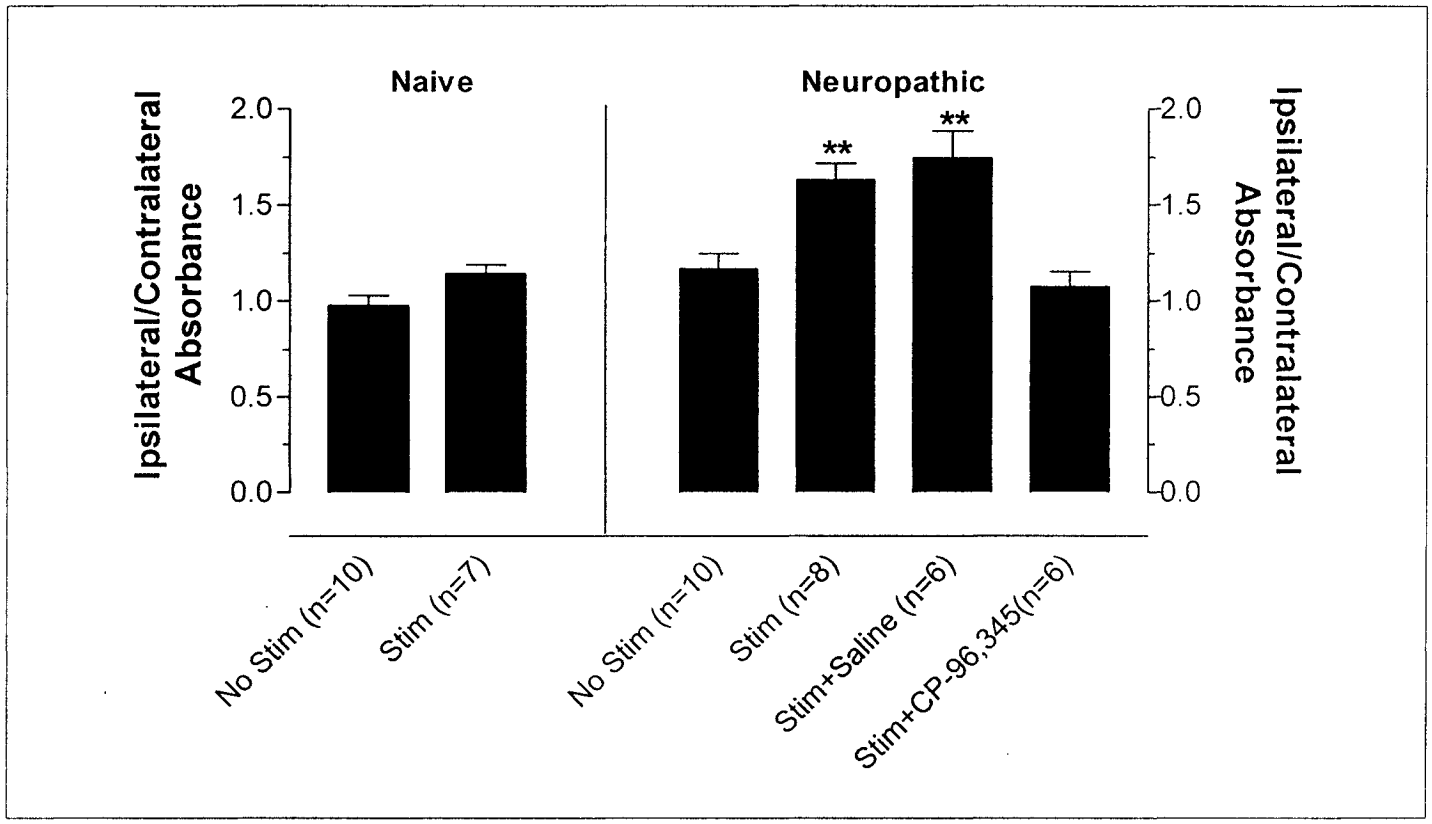

FIGURE 2. NK-1 receptors are activated following an innocuous peripheral stimulation. Animals were perfused 30 min after injection of Evan's Blue dye into the tail vein. Paws were subsequently cut immediately above the ankle, weighed and placed in 15ml of formamide at 60^{0}C overnight. After filtration, each vial's absorbance was measured by a spectrophotometer at wavelength of 620nm. Results were calculated according to the following formula:

[ipsilateral absorbance / ipsilateral weight] / [contralateral absorbance / contralateral weight]

Initially, four groups were examined: naïve, stimulated naïve, neuropathic, stimulated neuropathic. With a significant difference established between stimulated neuropathic animals versus all other groups, a blind trial of CP-96,345 and its vehicle saline were conducted. All results are shown on a single figure. Unpaired t-test revealed that both Neuropathic Stim and Stim + Saline treated animals were significantly different from all other groups, but not each other. No other groups were significantly different from one another. **$p<0.002$.

iii. Effect of CP-96, 345, morphine and other potential analgesics on mechanical allodynia in neuropathic animals

To begin examining the role of NK-1 receptors in neuropathic pain, the NK-1 receptor antagonist CP-96,345 was tested for its effect on mechanical allodynia in the Mosconi and Kruger model. Animals were administered CP-96,345 at an intraperitoneal (i.p.) dose of 5mg/kg and mechanical allodynia assessed by the application of von Frey filaments to the plantar surface of the ipsilateral paw. Unlike administration of the vehicle saline, the NK-1 receptor antagonist caused a highly significant increase in withdrawal threshold (Fig 3a). An increase was observed in all animals tested and five of the eleven animals returned all the way to pre-surgery baseline withdrawal thresholds.

Morphine was tested in the Mosconi and Kruger animal model in order to assess the efficacy of opioid analgesics commonly used to treat human neuropathic pain patients. Intraperitoneal administration of morphine (5mg/kg) caused a significant increase in withdrawal threshold (Fig 3b). The vehicle control, saline, had no effect on withdrawal responses. Although having a significant effect on mechanical allodynia, the elevated withdrawal threshold following morphine administration remained well below levels prior to nerve injury. Moreover, no single animal had a return to pre-surgical von Frey hair withdrawal threshold.

Intraperitoneal administration of gabapentin (50mg/kg) and intrathecal administration of phenytoin (36.5nmol), methylene blue (30μg), verapamil (50μg) or diltiazem (100μg) were all without significant effect when evaluated in this paradigm (Tbl. 1). There was a slight increase in withdrawal threshold in some animals following administration of gabapentin, however, it was not statistically significant (data not shown).

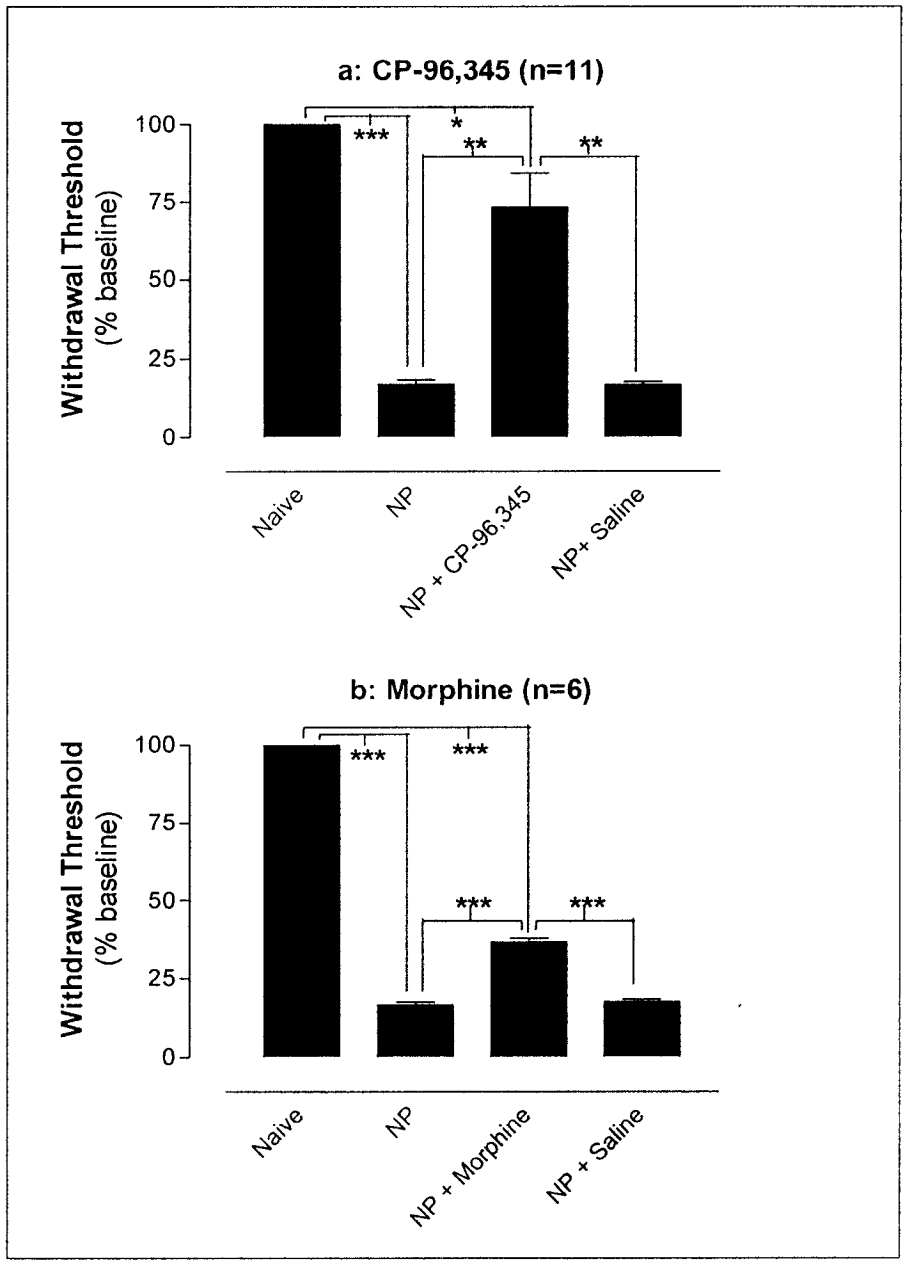

FIGURE 3. Both CP-96,345 and morphine partially alleviate mechanical allodynia in neuropathic animals. (a) Intraperitoneal administration of the NK-1 antagonist CP-96,345 (5mg/kg), significantly increases the mechanical withdrawal threshold of neuropathic animals, but saline vehicle does not. (b) To a lesser extent intraperitoneal administration of morphine significantly increases the mechanical withdrawal threshold of neuropathic animals, but saline vehicle does not. Mechanical allodynia is evaluated using in the von Frey hair test paradigm. Statistical analysis by paired t-tests revealed statistical significance, * p-value<0.05, ** p-value<0.005, *** p-value<0.0001. For both (a) and (b), there was no significant difference between Neuropathic and Saline treated animals.

Drug	Method of action	Dose	Route of administration	Result
Gabapentin	anticonvulsant-action unknown	50 mg/kg	intraperitoneal injection	no significant effect
Phenytoin	antiepileptic-action unknown	36.5 nmol	intrathecal catheter	no significant effect
Methylene Blue	guanylyl cyclase inhibitor	30 ug	intrathecal catheter	no significant effect
Verapamil	calcium channel blocker	50 ug	intrathecal catheter	no significant effect
Diltiazem	calcium channel blocker	100 ug	intrathecal catheter	no significant effect

TABLE 1. Effects of various drugs suspected of providing analgesia or currently employed in treating human neuropathic pains on mechanical allodynia in an animal model of neuropathic pain. Evaluated in von Frey hair testing paradigm. Dose is given per rat unless otherwise indicated.

iv. Effect of pretreatment with an intrathecally administered NK-1 receptor antisense oligonucleotide in preventing mechanical allodynia in neuropathic animals

Mechanical response thresholds (grams of force) were assessed by application of von Frey hairs to the plantar surface of the ipsilateral hind paw for all groups prior to sciatic nerve constriction surgery (baseline) and at various time points following nerve constriction (Fig 4a). Oligonucleotides (ODN) or artificial cerebrospinal fluid (ASCF) was administered for two days prior to surgery, and throughout the time course of testing, by continuous infusion via Alzet minipumps attached to intrathecal catheters for spinal delivery. Mechanical response thresholds were lower than baseline at all time points following sciatic nerve constriction in control animals (ACSF), indicating the induction of mechanical allodynia. Rats treated with antisense oligonucleotide (AS ODN) did not show a decrease in their mechanical response threshold when compared to baseline values. However, rats treated with mismatch (MIS) oligonucleotides had decreased mechanical withdrawal thresholds at all time points when compared to baseline values, similar to the results of the ACSF-treated group. In addition, the withdrawal threshold of the AS treated animals was significantly higher than both ACSF and MIS treated animals at all time points after cuff implantation. Mechanical withdrawal thresholds for the contralateral hind paw were not significantly different than baseline values indicating that the ODN treatment had no effect on normal thresholds. In non-surgical rats prolonged administration of ACSF, AS ODN or MIS ODN had no effect on mechanical response thresholds (data not shown). Therefore, only the antisense oligonucleotide was effective in preventing the onset of mechanical allodynia associated with neuropathic pain.

v. Effect of delayed intrathecal administration of NK-1 receptor antisense oligonucleotide on mechanical allodynia in neuropathic animals

Oligonucleotides or ASCF was administered for 72 h by continuous infusion via Alzet minipumps attached to intrathecal catheters for spinal delivery starting six days following nerve constriction (Fig 4b). Rats were deemed neuropathic by von Frey hair analysis prior to AS administration. In the ipsilateral paw of control animals (ACSF) the mechanical response thresholds were lower than baseline at all time points following sciatic nerve constriction indicating the induction of mechanical allodynia. Intrathecal administration of antisense reversed allodynia in the animal model of neuropathic pain at eight days after cuff implantation, after two days of antisense treatment whereas mismatch had no effect. ASCF, AS ODN or MIS treatment had no effect on mechanical response thresholds assessed in the contralateral hind paw. Therefore, the antisense oligonucleotide was able to relieve mechanical allodynia in previously neuropathic animals. Low dose (0.45 μg/μl/h) antisense ODN treatment had no effect on mechanical response thresholds when administered after onset of mechanical allodynia (data not shown) suggesting that the effects produced by AS were dose-dependent.

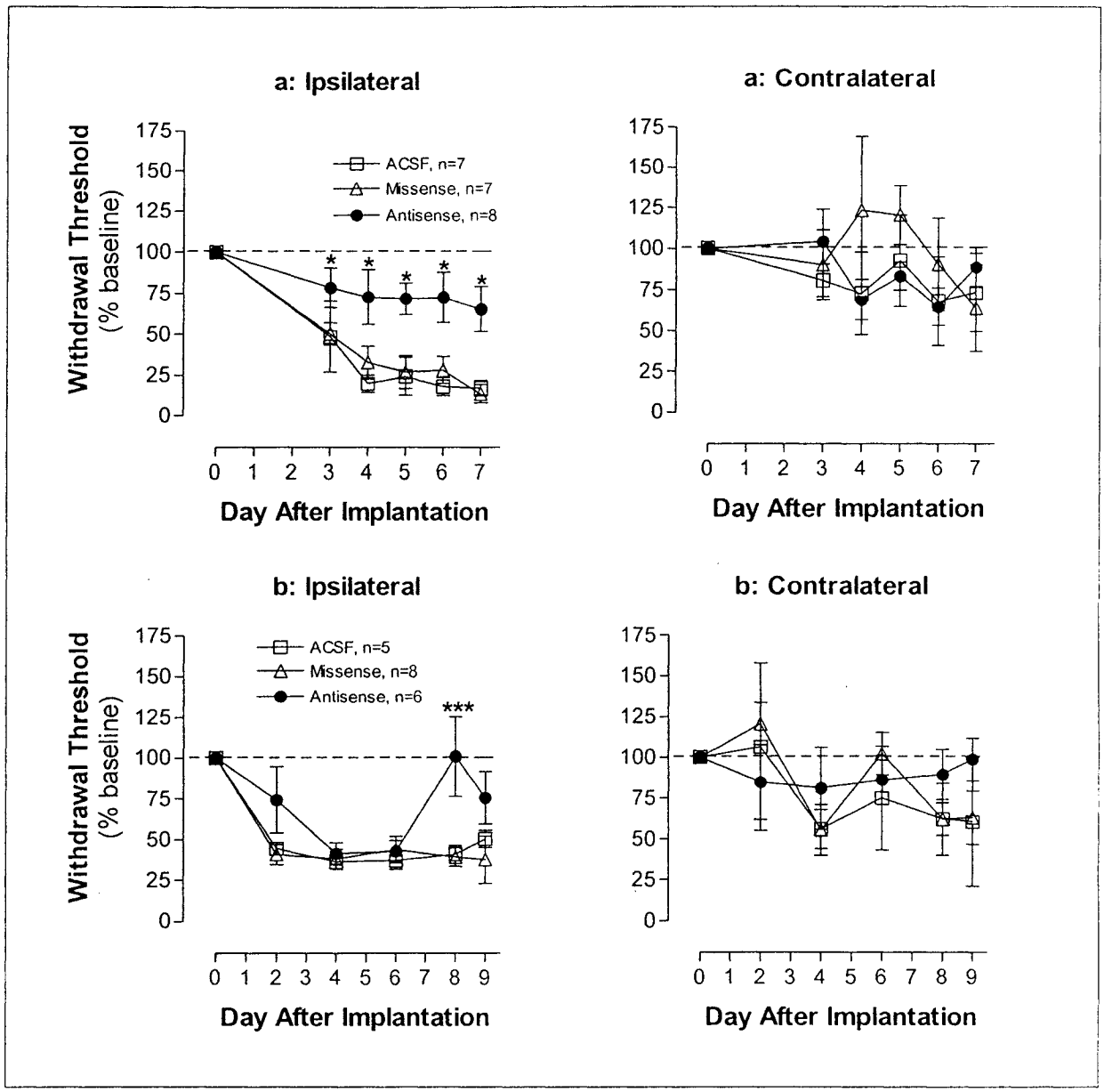

FIGURE 4. An NK-1 receptor antisense oligonucleotide delivered intrathecally significantly alleviates mechanical allodynia in neuropathic animals. (a) Treatment of the animals with the antisense oligonucleotide for two days prior to cuff implantation significantly retarded the characteristic development of mechanical allodynia following cuff implantation, *$p<0.025$ vs. missense, *$p<0.01$ vs. ACSF. Treatment with mismatch or artificial cerebrospinal fluid had no effect on the ipsilateral paw. The three treatments had no effect on the withdrawal threshold of the contralateral paw. (b) Treatment with the NK-1 receptor antisense oligonucleotide after mechanical allodynia had already been established following cuff implantation, significantly reversed the mechanical allodynia resulting from neuropathic surgery, ***$p<0.001$. Mismatch or artificial cerebrospinal fluid had no significant effect the ipsilateral paw. The three treatments had no significant effect on the withdrawal threshold of the contralateral paw. All testing was done within the von Frey hair test paradigm. Statistics were done by two-way ANOVA and post-hoc Tukey test.

vi. Effect of NK-1 receptor antisense oligonucleotide on substance P-induced thermal allodynia

To demonstrate the efficacy of the antisense in a physiological test, antisense (AS) or mismatch (MIS) ODNs as well as ACSF was administered intrathecally twice daily (75 μg/μl) for six days and tested for effect on substance P induced thermal allodynia in the tail-flick test. Testing was done prior to the first treatment then after three and six days of treatment. Baseline tail-flick latencies were assessed each day prior to intrathecal injection of substance P (SP, indicated by the arrows, Fig. 5). On day 0 (before oligonucleotide treatment had commenced) all animals yielded a decrease in tail-flick latency compared to baseline values following substance P (6.7 nmol/10 μl) administration indicating the occurrence of allodynia (Fig. 5). Three days after twice-daily injections of AS, substance P-induced thermal allodynia was significantly attenuated. Six days after AS treatment, there was a further decrease in the response to substance P. Statistical analysis using a two-way ANOVA revealed a significant difference between all testing days. Note that NK-1 receptor antisense treatment had no effect on baseline thresholds, indicating the lack of effect on acute nociceptive mechanisms. MIS ODN had no significant effect on the SP-induced thermal allodynia or on baseline thresholds at either three or six days of treatment. As expected ACSF administration was also without effect on tail-flick latency (data not shown).

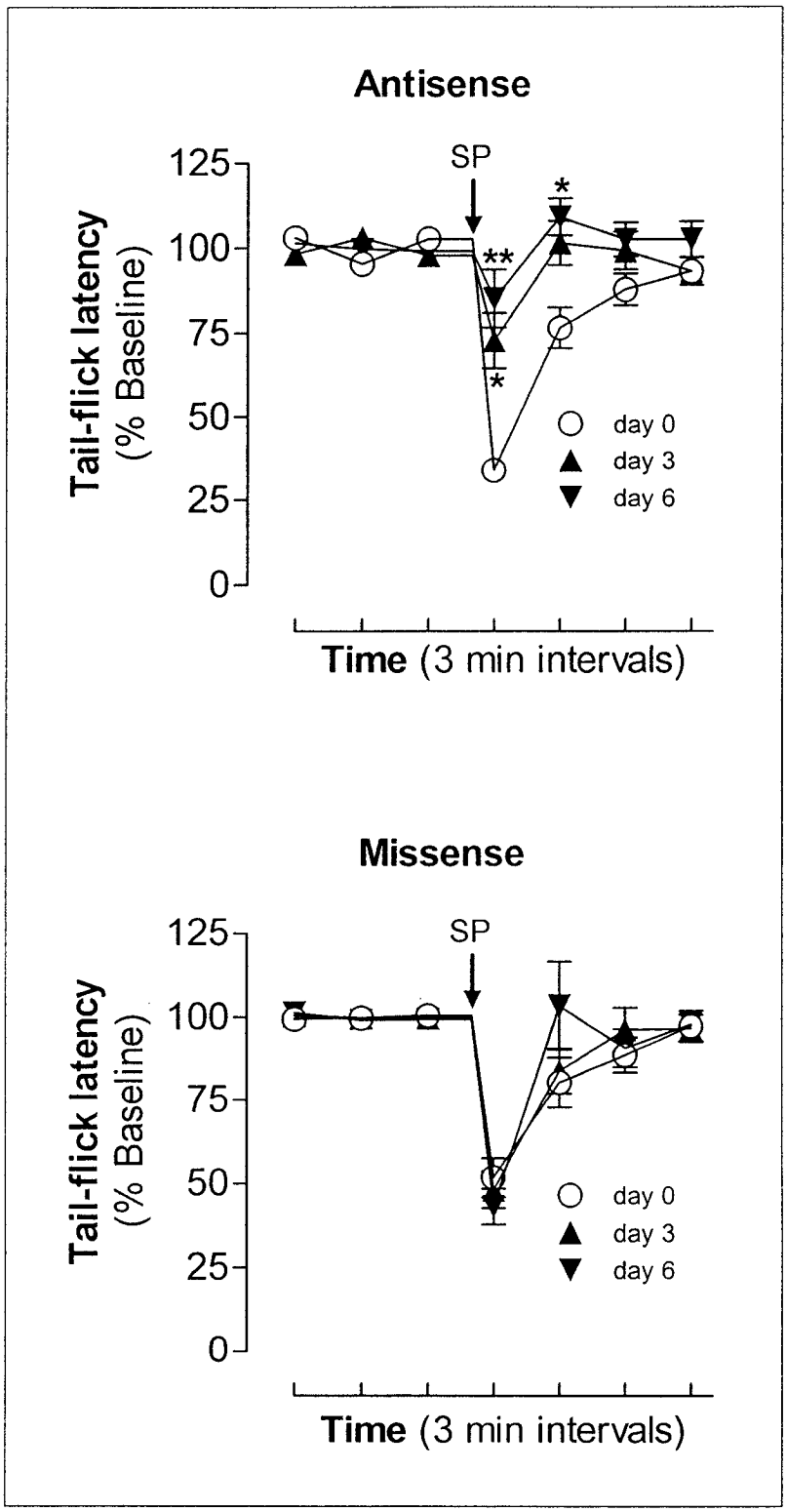

FIGURE 5. An NK-1 receptor antisense oligonucleotide attenuates substance P induced thermal allodynia in the tail-flick latency test. Prior to antisense treatment, intrathecal substance P administration causes a sharp transient decrease in tail-flick latency. This decrease is significantly reduced following three days of administration of the NK-1 receptor antisense oligonucleotide and is further reduced following six days of antisense treatment. Administration of missense oligonucleotide has no significant effect on tail-flick latency following substance P administration. Statistical analysis was performed by two-way ANOVA with post-hoc Tukey analysis using time and treatment as independent measures. The Tukey test revealed significant difference in antisense treated animals compared to control values at discrete time points. * p-value<0.05, ** p-value<0.005.

vii. Effect of intrathecal delivery of NK-1 receptor antisense oligonucleotide on NK-1 receptor protein expression and immunoreactivity

The efficacy of the antisense oligonucleotide (AS ODN) treatment was further demonstrated by decreased NK-1 receptor protein in western blotting experiments from polyacrylamide gel electrophoresis of spinal cord membranes (Fig 6). ODNs or artificial cerebral spinal fluid (ASCF) were administered for 48 h prior to sciatic nerve constriction and continuously for six days (2 μg/μl/h) via Alzet minipumps attached to intrathecal catheters for spinal delivery. Membranes from all groups showed an NK-1 receptor protein immunoreactive band at approximately 50 kDa, which agrees with the reported molecular weight for the NK-1 receptor by other studies (Macdonald et al., 1996; Van Ginkel and Pascual, 1996). The density of immunoreactivity of the band was decreased in samples from antisense-treated animals compared with either mismatch or ACSF treatment, which exhibited equally prominent bands.

The efficacy of the antisense oligonucleotide administration was again demonstrated by NK-1 receptor immunoreactivity in lumbar spinal cord. In mismatch treated animals, NK-1 receptor immunoreactivity was not different from naïve tissue (not shown) and was similar to the distribution reported previously (reviewed by Ribeiro-Da-Silva and Hokfelt., 2000). NK-1 staining was mostly restricted to lamina I with a moderate level of staining in lamina III-V (Fig 7). NK-1 receptor immunoreactivity was evident in neuronal cell bodies and dendrites. NK-1 receptor immunoreactivity was significantly attenuated in NK-1 antisense treated rats compared to mismatch, whereas substance P immunoreactivity was identical in both groups. Merged images demonstrate the overlap of substance P and NK-1 receptor immunoreactivity in both groups.

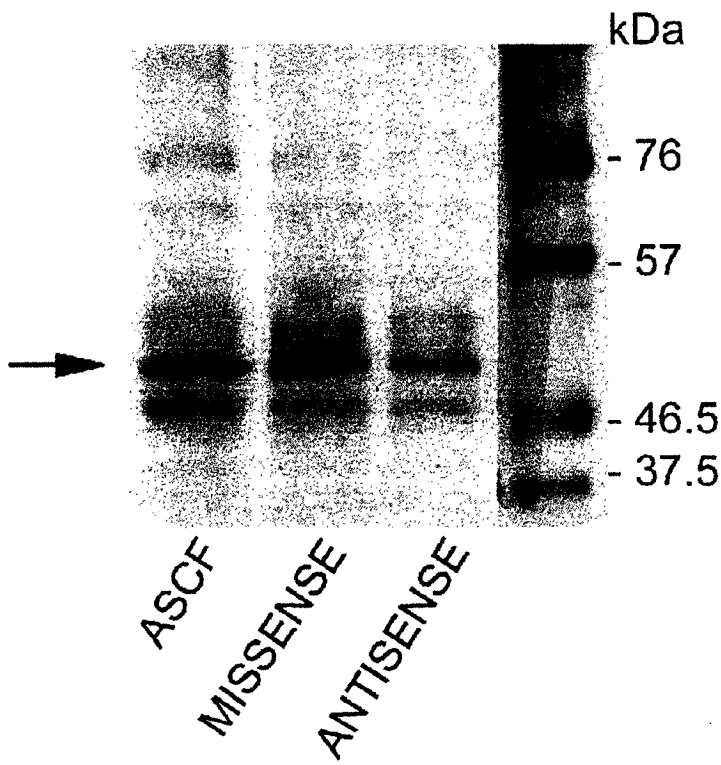

FIGURE 6. An NK-1 receptror antisense oligonucleotide decreases immunoreactivity of NK-1 receptor protein. A western blot from polyacrylamide gel electrophoresis of spinal cord membranes from animals treated with ACSF, MIS, or AS. There is little difference between prominent bands of immunoreactivity for ACSF and MIS animals however, a significant decrease is visible in the residual band for AS animals.

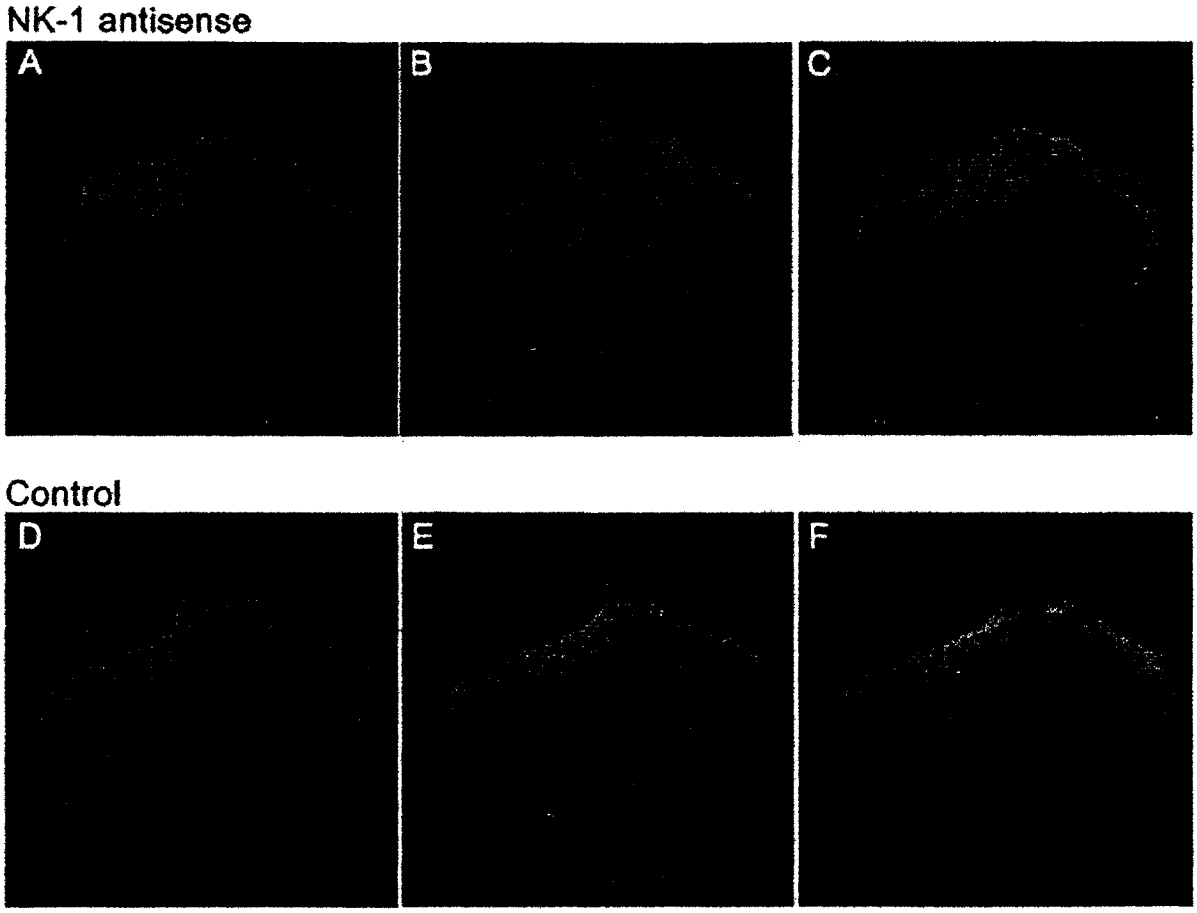

FIGURE 7. Immunofluorescent labelling of NK-1 and substance P in the dorsal horn of the lumbar spinal cord indicate a decrease in NK-1 receptor expression in antisense treated animals. 7A illustrates substance P staining in antisense treated animals, which is not decreased compared to controls 7D. 7B shows decreased NK-1 staining due to antisense treatment as compared to controls 7E. 7C and 7F are merged images of 7A & 7B and 7C & 7D, respectively. The merged images illustrate the co-expression of substance P and NK-1 receptors.

viii. Mass spectrum of the lumbar spinal cord of neuropathic and naïve animals

Collaborations with MDS Pharma Services Inc. allowed the assessment of substance P levels in the spinal cord of neuropathic versus control animals. Pure substance P in the mass spectrometer produced a doubly-charged peak at 674.3922 (not shown), as expected since the molecular weight of substance P is 1347.71. The mass spectrum of the lumbar spinal cord of neuropathic animals revealed a large doubly-charged peak at 674.3795, indicative of substance P (Fig 8a). Control animals did not have any significant peak at this point (Fig 8b).

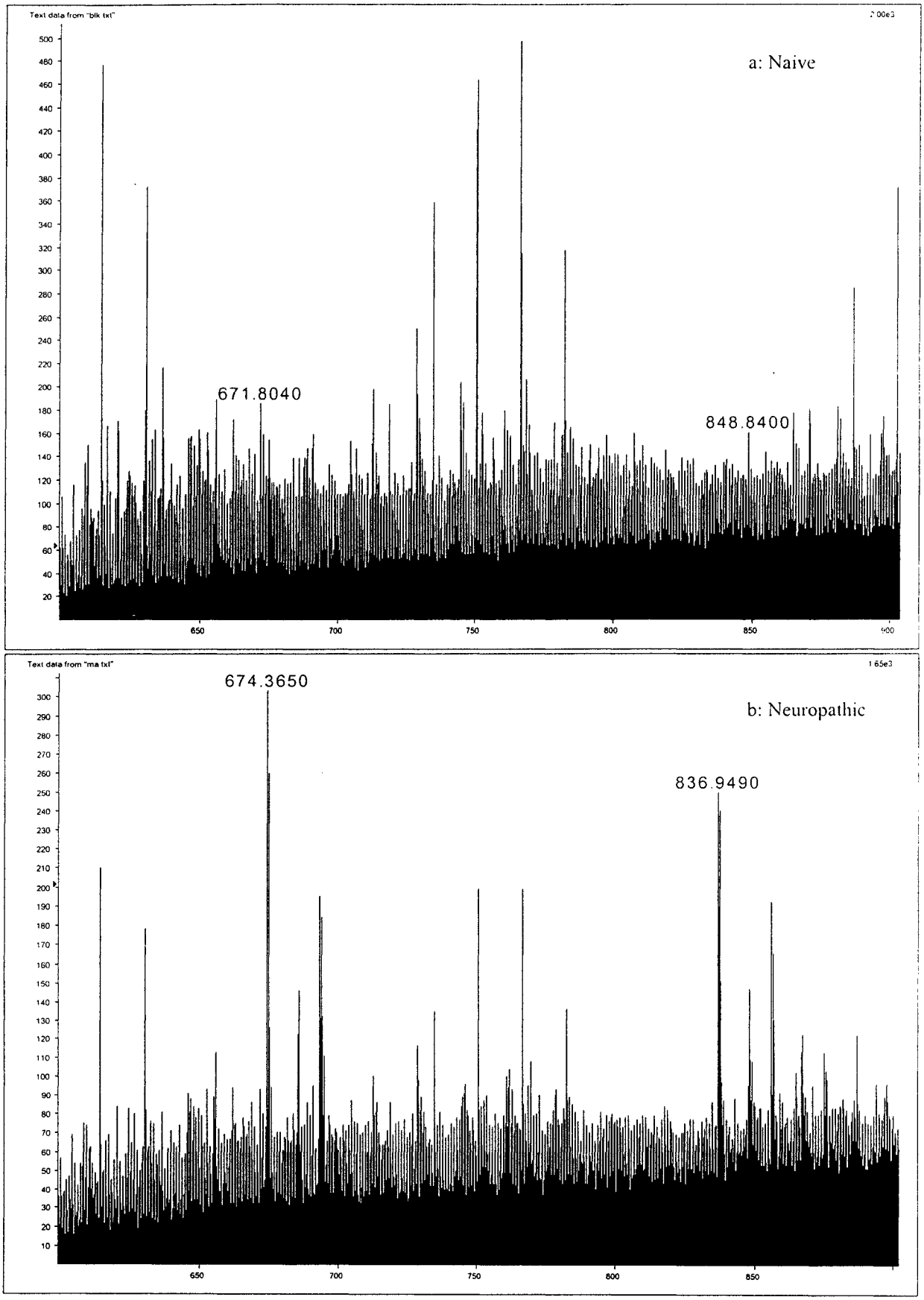

FIGURE 7. Quantification of substance P in the lumbar spinal cord by mass spectrometry. Naive animals (a) show a small doubly-charged peak for substance P, while neuropathic animals (b) show a dramatically increased peak.

V. Discussion

In this study, a series of experiments were conducted using the Mosconi and Kruger rat model of neuropathic pain investigating the involvement of substance P and the NK-1 receptor in this painful pathology. Our initial investigations demonstrated a clear activation of NK-1 receptors following an innocuous peripheral stimulation of the ipsilateral hind paw of neuropathic but not naïve animals. Since NK-1 receptors are known to be involved in the transmission of pain information, we examined the effect of blocking the receptor with an antagonist and of downregulating receptor expression with a functional antisense oligonucleotide. In this model, both approaches to stopping NK-1 receptor activation significantly reduced the characteristic mechanical allodynia more effectively than the other therapeutic alternatives that we tested. Furthermore, we sought an alternative approach to quantify substance P in the spinal cord. Through mass spectrometric analysis of the lumbar spinal cord of neuropathic and naïve animals, we observed a dramatic increase in substance P in neuropathic animals only. Taken together, the data suggest a primary role for substance P activating the NK-1 receptor in the mechanical allodynia seen in neuropathic animals. The study justifies the examination of substance P and NK-1 receptor blocking agents in human neuropathic pain patients who do not obtain useful analgesia using current therapies.

i. NK-1 receptors are activated following a normally innocuous stimulus

In the dorsal horn, the NK-1 receptor is bound by the ligand, substance P, and elicits slow excitatory action potentials on second-order nociceptive-specific neurons (De Koninck and Henry, 1991; Radhakrishnan and Henry, 1991). Under normal conditions, substance P is released from small caliber primary afferents only following a noxious stimulus (Duggan and Hendry, 1986; Brodin et al, 1987). However, earlier investigations have

shown that NK-1 receptor antagonists can significantly relieve allodynia in neuropathic animals exhibiting painful responses to low threshold stimuli (Cumberbatch et al., 1998; Coudore-Civiale et al., 1998; Campbell et al., 1998; Gonzalez et al., 2000; Cahill and Coderre, 2002). Alterations in peptide expression of nerves may occur as an adaptive response to injury (Hokfelt et al., 1994). Moreover, touch-sensitive A-beta fibers may undergo a phenotypic change allowing the production of pain mediators such as substance P (Neumann et al., 1996). Furthermore, two weeks after neuropathic surgery, isolated A-beta fibers release substance P upon electrical stimulation (Malcangio et al., 2000). The fact that touch sensitive fibers could be capable of releasing substance P onto the superficial dorsal horn led us to develop investigational approaches to determine if NK-1 receptors are activated by a light tactile stimulus in neuropathic animals.

It has repeatedly been shown that high intensity stimulation of nerves projecting to one spinal segment can produce antinociception at another elsewhere along the spinal cord (Laird and Cervero, 1989, Fleischmann and Urca, 1989; Alarcon and Cervero, 1990; Ness and Gebhart, 1991, 1991). For example, a noxious peripheral stimulation of a rat hind paw produces antinociception in the tail causing increased latency in the tail-flick test (Yashpal et al., 1995). The endogenous antinociception or heterosegmental inhibition may be blocked by an NK-1 receptor antagonist or naloxone, implicating NK-1 and opioid mechanisms (Yashpal et al., 1995). We hypothesized that the chemicals involved in the response to a noxious stimulus may also be present following innocuous stimulation of a neuropathic animal, given that NK-1 receptors seem to be involved in the mechanical allodynia of neuropathic animals. Therefore, we investigated the possible activation of NK-1 receptors after an innocuous peripheral stimulation. Our first

approach was to look for substance P (NK-1) mediated heterosegmental inhibition in the tail-flick test.

A light tactile stimulus was applied to the plantar surface of the ipsilateral paw of the neuropathic animals and effects observed in the tail-flick test. This method of stimulation was selected as it would cause an allodynic pain sensation in human neuropathic conditions such as postherpetic neuralgia, but is undoubtedly non-painful in normal subjects. Indeed, an innocuous stimulation of the ipsilateral hind paw has been shown to produce an afterdischarge in electrophysiological experiments, which can be blocked with an NK-1 receptor antagonist (Pitcher et al, in print). To the best of our knowledge, the activation of NK-1 receptors in neuropathic animals due to an innocuous stimulus has not been previously demonstrated *in vivo* through behavioral testing of awake animals.

Naïve animals showed a slight decrease in latency following the first handling and an additional decrease following stimulation, despite extensive conditioning to the experimental paradigm (Fig 1). The purpose of the initial handling without stimulation was to ensure that any changes observed could be distinguished as strictly due to the innocuous stimulation of the paw and not merely the handling of the animal. The pattern was seen quite clearly in all animals and was thus deemed a physiological effect of removal from the apparatus. No further investigation as to the cause of this effect was undertaken.

In neuropathic animals the innocuous peripheral stimulation caused a sharp, transient increase in tail-flick latency. Thus, the stimulation had an antinociceptive effect on the latency to tail removal. Our interpretation of the data is that an innocuous peripheral stimulation had some effect activating endogenous antinociceptive mechanisms, which would normally occur only following a noxious stimulus.

As earlier evidence implicated substance P and the NK-1 receptor in heterosegmental inhibition following a noxious stimulus and an innocuous stimulus seemed to elicit a similar antinociceptive response in neuropathic animals, we examined the effects of an the NK-1 antagonist CP-96,345. The result was a complete abolition of the antinociceptive effect. In fact, the results were similar to those seen in the naïve animals. We concluded that the transient spike of heterosegmental inhibition was the result of the activation of NK-1 receptors, most likely by the tachykinin substance P. Therefore, an innocuous peripheral stimulation of the ipsilateral hind paw of neuropathic animals can lead to the activation of NK-1 receptors, which would not occur in normal animals.

A peripheral stimulus triggers the release of chemicals contained in nerve terminals centrally and by antidromic stimulation the same chemicals peripherally. Moreover, noxious peripheral stimulation of a rat's hind paw causes substance P release and subsequent binding and activation of NK-1 receptors both centrally and peripherally. The diverse effects of substance P released peripherally into surrounding tissues include vasodilatation and enhanced vascular permeability with resulting plasma extravasation (Hartung and Tokya, 1989). These observations are part of a larger effect known as neurogenic inflammation. Since the innocuous brush stimulus employed in our earlier experiments seemed to cause the activation of NK-1 receptors, we proceeded to investigate the effect of a similar stimulus on peripheral plasma extravasation.

Initial investigations examined plasma extravasation in the paws of naïve and neuropathic animals with and without the innocuous peripheral stimulation of the plantar surface of the ipsilateral paw. The absorbance for each paw was divided by its wet weight to avoid any discrepancies due to size. Also, the ipsilateral absorbance was divided by the contralateral absorbance to provide an internal control for each animal.

All naïve animals had an overall result around 1.0 indicating that there was no difference in plasma extravasation in the two paws, with or without stimulation (Fig 2). Likewise, unstimulated neuropathic animals did not differ between the ipsilateral and contralateral paws. In the ipsilateral paws of stimulated neuropathic animals however, there was a sharp increase in plasma extravasation relative to the contralateral side. A subsequent investigation examined the effects of systemically administered saline and the NK-1 antagonist CP-96,345 on this increase. While saline was without effect, CP-96,345 significantly reduced plasma extravasation in the ipsilateral paw of the stimulated neuropathic animals. The results indicate that an innocuous peripheral stimulation of the ipsilateral paw of neuropathic animals leads to the activation of NK-1 receptors, which are involved in inducing plasma extravasation. Due to the nature of the peripheral plasma extravasation, we surmised that the effect was due to the release of substance P.

Few studies have examined substance P induced neurogenic inflammation in neuropathic animals. One study reported a decrease in the plasma extravasation and vasodilatation response to substance P in neuropathic animals (Basile et al, 1993). A more recent publication demonstrated an increase in the basal release of substance P in neuropathic animals eventually causing a degree of desensitization leaving animals less responsive to exogenously administered substance P, apparent seven days after surgery (Yonehara and Yoshimura, 2001). This does not contradict our results, as the increase in plasma extravasation we report here, though significant, is not extreme, perhaps due to the desensitization of the receptors rather than a minimal quantity of substance P released.

In their experiments Yonehara and Yoshimura took subcutaneous fractions of perfusate and examined substance P content. They note in their discussion that neuropathic, but not control animals, had a major release of substance P in the first fractions of each perfusion.

They equate the gentle perfusion pressure to a mild mechanical stimulation. This is in agreement with our results, which show that a light tactile stimulation is able to cause activation of peripheral NK-1 receptors. We conclude that an innocuous peripheral stimulation is able to induce plasma extravasation in part due to the activation of NK-1 receptors, presumably by the antidromic release of substance P.

ii. NK-1 receptors are involved in the onset and maintenance of mechanical allodynia

Having determined that NK-1 receptors are activated following an innocuous mechanical stimulus, we examined whether these nociceptors contribute to the mechanical allodynia of neuropathic animals. There has been some disagreement as to the involvement of substance P and NK-1 receptors in neuropathic pains. In the chronic constriction injury (CCI) model (Bennett and Xie, 1988), systemic administration of the NK-1 receptor antagonist GR205171 blocked the receptive field expansion of dorsal horns and decreased mechanical allodynia (Cumberbatch et al., 1998). Another NK-1 receptor antagonist SR-48,968 reduced vocalizations from mechanical stimuli in CCI and diabetic rats (Coudore-Civiale et al., 1998). Other studies have reported that different NK-1 receptor antagonists in various animal models of neuropathic pain have anti-allodynic or anti-hyperalgesic effects (Campbell et al., 1998; Gonzalez et al., 2000; Cahill et al., 2002). Furthermore, transgenic mice lacking the NK-1 receptor that are rendered neuropathic do not display the painful behavior in response to an innocuous stimulus (Mansikka, 2000). However, mechanical allodynia in substance P/NK-A knockout mice rendered neuropathic was not significantly different from wild-type (Cao et al., 1988). Also, the antagonist RP 97530 was reported to be ineffective in the treatment of diabetic neuropathy in rats (Malcangio and Tomlinson, 1998). Most likely these differences are due to methodological and

model differences. Nonetheless, it is necessary to reexamine the role of NK-1 receptors in mechanical allodynia through more than one approach.

When tested in the Mosconi and Kruger model, CP-96,345 and morphine both significantly alleviated mechanical allodynia as assessed by von Frey hair analysis, while their vehicle saline was without effect (Fig 3). CP-96,345 was considerably superior to morphine, however, neither drug was able to fully alleviate the allodynia. Intrathecally administered calcium channel blockers verapamil and diltiazem were both ineffective in alleviating the neuropathic pain. Similarly, the guanylyl cyclase inhibitor, methylene blue, and the antiepileptic drug, phenytoin, were unsuccessful. The anticonvulsant gabapentin, which is clearly effective in some human neuropathic pains, had a small, but statistically insignificant effect. These results emphasize the significance of the analgesia provided by the NK-1 receptor antagonist in this animal model of neuropathic pain as CP-96,345 was considerably more effective than other currently employed analgesics.

In an effort to develop a novel treatment for neuropathic pain and confirm the importance of the NK-1 receptor, we developed an antisense oligonucleotide (AS ODN) to downregulate the receptor's mRNA. To physiologically test the efficacy of the AS ODN, we administered substance P intrathecally to animals treated with artificial cerebrospinal fluid (ACSF), AS ODN or mismatch oligonucleotide (MIS ODN) and tested their tail-flick latency (Fig. 6). Previous studies have shown that intrathecal substance P causes decreased tail-flick latency indicative of thermal allodynia, which can be blocked with an NK-1 receptor antagonist (Yashpal et al., 1982; Yashpal et al., 1993). Significant decreases in the response to substance P administration occurred after three days and further after six days of AS ODN treatment indicating a downregulation of NK-1 receptor expression. As expected no changes in thermal allodynia occurred after three or six days

of MIS ODN treatment. Western Blotting experiments to further examine the functionality of our AS ODN, showed a less dense immunoreactive band for AS ODN treated animals compared to missense (MIS) and artificial cerebrospinal fluid (ACSF) treated animals (Fig 5). Therefore, the AS was effective in downregulating the NK-1 receptor, but the knockdown was not complete. Although we cannot explain the presence of a residual immunoreactive band for AS ODN treated animals, we propose possibilities. The AS could act only to knockdown the functional surface receptors required for a physiological impact, while the Western exposes all intracellular NK-1 receptors as well. Alternatively, the requirement for physiological efficacy may be only a percentage of the total population of receptors. Previous studies using AS ODNs have also reported significant physiological effects without complete elimination of the protein. Additional immunohistochemical studies demonstrated a decrease in NK-1 immunoreactivity without any change in substance P. The AS ODN was therefore deemed functional and examined for effect in the treatment of neuropathic pain.

Administration of the NK-1 antisense oligonucleotide two days prior to cuff implantation or after the inception of neuropathic pain blocked the onset and significantly attenuated established mechanical allodynia, respectively. These antisense results are consistent with our antagonist result indicating that stopping activation of NK-1 receptors is analgesic in neuropathic pain. In addition to blocking the NK-1 receptor, CP-96,345 is known to have effects on T-type calcium channels. The results of the antisense oligonucleotide indicate that the analgesic effect of the antagonist is primarily due to blocking the NK-1 receptor rather than any action on calcium channels.

Despite the substantial analgesia provided by the NK-1 receptor antagonist or the NK-1 AS OND, neither granted complete analgesia indicating that there is likely one or more

mechanism other than that of NK-1 activation creating and maintaining the neuropathic condition. Nonetheless, the significant analgesic effects demonstrated with the NK-1 antagonist and the NK-1 antisense oligonucleotide confirm that the receptors are of functional importance in neuropathic pain.

iii. Possible involvement of substance P

Substance P has long been thought to be the preferred tachykinin for the NK-1 receptor. Intrathecal administration of substance P causes hyperalgesia in the tail-flick test, which can be blocked by an NK-1 receptor antagonist (Yashpal et al., 1982; Yashpal et al 1993). Also, electrophysiological evidence shows that iontophoretic application of substance P on the dorsal horn causes firing of secondary nociceptive neurons via activation of NK-1 receptors (De Koninck and Henry, 1991; Radhakrishnan and Henry, 1991). Moreover, upregulation of NK-1 receptors (Aanonsen et al., 1992; Goff et al., 1998) and analgesic efficacy of NK-1 receptor antagonists in animal models of neuropathic pain seem to implicate substance P as the receptor's main ligand.

Although substance P immunoreactivity has been reported to decrease in some animal models of neuropathic pain (Cameron et al., 1991; Garrison et al., 1993; Munglani et al., 1995), others have reported nerve injury to cause increases in substance P mRNA in the dorsal horn (Delander et al., 1997). In addition, increases in preprotachykinin-A mRNA in the dorsal root ganglion (Marchand et al., 1994; Noguchi et al., 1994) have been reported, which are thought to be reflective of phenotypic changes in large diameter A-beta fibers as they begin to express substance P (Neumann et al, 1996).

Here we undertake a novel approach to examining oligopeptides in the lumbar spinal cord of naïve and neuropathic animals. A mass spectrum analysis was completed revealing substance P in the samples from neuropathic rats, which were significantly smaller in

control animals. This result indicates a dramatic upregulation of substance P seven days after cuff implantation.

Complicating the NK-1/substance P issue is the growing number of studies reporting approximately equal affinities of substance P and NK-A for the NK-1 receptor. In fact, NK-1 receptor internalization, indicative of ligand binding, is reported to be at least 50% caused by NK-A following a noxious stimulation in a normal animal (Trafton et al., 2001). Our study is not sufficient to disprove the involvement of NK-A however, the mass spectrum analysis revealed no evidence of upregulation of NK-A in the lumbar region, only substance P. Therefore, without discounting the possible involvement of NK-A, substance P may be the foremost NK-1 receptor ligand in our model.

iv. Conclusions

The data presented in this study demonstrate that activation of NK-1 receptors in neuropathic rats can occur following a normally innocuous stimulus. Moreover, that mechanical allodynia can be significantly alleviated by blocking the activation of NK-1 receptors by chemical mediators such as substance P, which seems to be upregulated in this model. Therefore, it would be justified to reexamine the use of agents that inhibit the activation of NK-1 receptors in the treatment of drug resistant neuropathies.

SUMMARY AND CONCLUSIONS

The primary aim of this study was to determine the importance of NK-1 receptors in neuropathic pain. Additionally, the goal was to investigate substance P and NK-1 receptors as potential targets for the development of novel analgesics for the treatment of neuropathies. The Mosconi and Kruger model was selected as we felt it provided the most consistently reproducible characteristic mechanical allodynia of the available rat models of neuropathic pain.

Our initial investigations focused on determining whether an innocuous stimulus could instigate the activation of NK-1 receptors, normally involved in the transmission of nociceptive information. We felt this was important as it could mean simple movements trigger a similar activation. In our studies, an NK-1 receptor antagonist successfully blocked heterosegmental inhibition in the tail-flick test as well as increased plasma extravasation in the paw, which were caused by an innocuous tactile stimulation of the ipsilateral hind paw. To the best of our knowledge, this is the first time an innocuous stimulus has been shown to activate NK-1 receptors *in vivo* in neuropathic animals. Furthermore, we believe this is the first time endogenous antinociception or increased plasma extravasation in the paw has been reported as a result of such a stimulus.

The efficacy of an NK-1 antagonist in treating mechanical allodynia is not a novel concept. However, the results of the antisense oligonucleotide lend a great deal more credibility to the importance of NK-1 receptor activation in the perception of mechanical allodynia. To the best of our knowledge this is the first study reporting the use of a

functional NK-1 antisense oligonucleotide or an upregulation of substance P evaluated by mass spectrometry.

Following nerve injury spinal reorganization may occur as C-fibers degenerate from lamina II and subsequently A-beta fibers project into the vacancy (Woolf et al., 1992). Also, as mentioned, A-beta fibers may be phenotypically altered to produce substance P (Neumann, 1996), and can be electrically stimulated to release substance P *in vitro* (Malcangio et al., 2000). In combination, these changes could allow touch-sensitive fibers to release substance P onto the superficial dorsal horn where NK-1 receptors on nociceptive secondary neurons could be activated. The phenotypic changes in A-beta fibers could explain the increased substance P we report in the lumbar spinal cord despite the decrease in substance P containing C-fibers, and furthermore, account for the activation of NK-1 receptors following an innocuous tactile stimulation. Finally, this release of substance P from innocuously activated A-beta fibers, would account for the efficacy of an NK-1 receptor antagonist and a similarly targeted antisense oligonucleotide in the amelioration of allodynia.

These data strongly implicate the NK-1 receptor's activation as a fundamental element of the initiation and immediate maintenance of neuropathic pain, and suggest that substance P is the foremost ligand for the receptor in this condition. Taken together, the results in this study justify the reexamination of NK-1 antagonists or other approaches to interfere with the activation of NK-1 receptors as novel treatments for human neuropathic pains for which current analgesics are inadequate.

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APPENDIX